Section 1: Vector Biology and Ecology

NON-CULTURE DEPENDENT SURVEY OF THE MICROBIOTA OF THE GLASSY-WINGED SHARPSHOOTER USING 454 PYROSEQUENCING

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ABSTRACT

The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) is an invasive pest that has spread across the southern and western United States. It is highly polyphagous, feeding on at least 100 species in 31 families (Hoddle et al., 2003; Turner and Pollard, 1959), and a voracious feeder, having been known to consume up to 100 times its weight in xylem fluid daily. This insect is a vector of the phytopathogen *Xylella fastidiosa* (*Xf*), which is the causative agent of Pierce's disease (PD) in grapevines. In order to evaluate the microbial flora associated with GWSS hemolymph, alimentary canal excretions and whole insect bodies were subjected to 16S pyrosequencing using the bTEFAP methodology and the resulting sequences (370-520 bp) were compared to a curated high quality 16S database derived from NCBI's GenBank. Species from the genera *Wolbachia*, *Delftia* (formerly *Pseudomonas*), *Pectobacterium*, *Moraxella*, *Serratia*, *Bacillus* and many others were detected and a comprehensive picture of the microbiome associated with GWSS was established. Some of the bacteria identified in this report are initial discoveries and having a breadth of knowledge as to the microbial flora of this insect pest can serve as a reservoir of information for developing biological control strategies. One method for biological control can be the genetic engineering of a particular bacterium to deliver certain molecules known to affect the life stage development of an insect. Another method, could be isolating a bacterium that competes with *Xf*, and re-delivering it to wild populations in excess of their natural bacterial load. Within this study, we have identified the types of bacteria which may be ubiquitous among GWSS providing us with targets to begin to investigate these future directions.

LAYPERSON SUMMARY

The glassy-winged sharpshooter is an insect pest that spreads the bacterium *Xylella fastidiosa*, the causal agent of Pierce's disease in the grapevine. Both wine and table grape production is affected by this disease and it has become a major financial burden on the industry. Bacterial DNA can be used to screen for such pathogens in insect populations and having knowledge of the bacterial pathogens of the insect can be used to develop a biological control strategy. Hemolymph, alimentary canal excretions and whole insect tissue were subjected to DNA sequencing and the resulting sequences were matched to groups of bacteria using NCBI's GenBank database. This study shows that bacteria such as *Wolbachia*, *Delftia*, *Pectobacterium*, *Moraxella*, *Serratia*, and *Bacillus* spp may be useful targets to develop biological control strategies.

INTRODUCTION

The glassy-winged sharpshooter (GWSS) is a highly mobile pest and transmits the xylem-limited bacterium *Xylella fastidiosa (Xf)*. This bacterium can cause disease in many economically important plants including the grapevine, peach, and citrus and has become a major limiting factor in their mass production. The bacterium can also cause disease in ornamentals such as the oak, elm and sycamore. Once a plant has become infected with *Xf*, it becomes a reservoir for bacterium and can be easily spread from plant to plant by the near-continuous feeding of GWSS. Although pesticides are available for the control of the insect, resistance and harm to non-target insects is an issue of concern. Naturally occurring forms of control have been successful and should be further pursued as a complimentary strategy for insect and disease control.

Many insect taxa have obligate endosymbionts that supplement nutrition in exchange for vertical or horizontal transfer among individuals (Moran et al 2005, Buchner 1965). This mutualism has allowed insects to occupy or thrive in otherwise hostile niches. The GWSS, a xylem feeder, is known to host several bacterial species including *Baumannia cicadellinicola* and *Sulcia muelleri* (Wu et al 2006). The *B. cicadellinicola* genome is devoted to the biosynthesis of vitamins and cofactors but lacks most amino acid biosynthetic pathways, whereas *S. muelleri* apparently produces most of the amino acids needed for the host. DGGE has been used to find other symbiotic bacteria including *Wolbachia*, *Bacillus*, *Pseudomonas*, *Pedobacter*, *Methylobacterium,* and *Curtobacterium flaccumfaciens* which the authors suggest could be used as forms of symbiotic control (Lacava et al 2007). Curly et al (2007) identified bacteria closely related to *Stenotrophomonas* and *Acinetobacter* in hemolymph samples.

OBJECTIVES

1. Identify major groups of bacteria in the hemolymph, alimentary canal and whole insect.
2. Identify species of bacteria for possible transgenesis and biological control.

RESULTS AND DISCUSSION

Using 16S pyrosequencing based upon the bTEFAP methodology (Dowd et al., 2008a; Dowd et al., 2008b) optimized for the Titanium pyrosequencing platform (Roche, Indianapolis, IN) , we were able to identify 17 orders (**Figures 1-3**), 28 families and at least 38 genera (**Figures 4-6**) of bacteria. Sequences were taken from separately prepared extracts of hemolymph, alimentary canal excretions and macerated whole insects suspended in 1X PBS. The sequences were approximately 500 bp (370-520 bp) and were compared to NCBI's basic local alignment search tool (BLAST) for homologies. Some of the shorter sequences aligned to multiple genera and were placed in a separate category called "Other" because it was not clear which identification was appropriate.

The hemolymph extracts (**Figures 1 and 4**) contained over 1000 sequences aligning with the order Enterobacteriales although no *Enterobacter* were found at the genus level. Up to 27 sequences from Burkholderiales were found in the hemolymph but no *Burkholderia*, *Bordetella* or *Oxalobacter* related sequences were found at the genus level. A single sequence aligning with Rhizobiales and two sequences aligning with *Clostridium* were also found in the hemolyph.

The alimentary canal excretions (**Figures 2 and 5**) contained Enterobacteriales related sequences. This coupled with the Enterobacteriales found in the hemolymph may be a sign of cross-contamination with respect to preparation of samples. The hemolymph vessels and the alimentary canal lie close to one another in the body of the insect and may have been punctured in some trials. Thirteen sequences from *Bacillus*, 52 *Moraxella* (an opportunistic cattle and human pathogen) and 72 Serratia (a human pathogen and lab-colony limiting agent) were recovered as well.

Whole insect macerations (**Figures 3 and 6**) contained 74 sequences related to *Wolbachia*, a well-known intracellular insect pathogen that has been characterized in previous studies. This symbiont has been shown to be obligate in many arthropods and nematode species (Mavingui et al 2005) and may be a target for limiting populations of GWSS. Sequences related to *Cardiobacterium* spp. were also recovered in large numbers from all but hemolymph samples. It is not clear why this particular bacterium is present.

Sequences of *Pectobacterium* were recovered from all extracts of the glassy-winged sharpshooter. This bacterium is known to cause soft rot and black leg in potato plants through its arsenal of extracellular pectinases (Chan et al 2009). This identification is believed to be the first report of this phytopathogen in GWSS.

Although not clearly understood at this moment, sequences relating to *Delftia* sp were only recovered from the hemolymph extracts. *Delftia* sp are ubiquitous, rod-shaped, gram-negative bacteria (Hai et al 2007) that are able to degrade di-n-butylphthalate (DBP), an industrial pollutant and phthalate derivative, as a sole source of carbon and energy (Neelakanteshwar et al 2006).

Fifty two (52) sequences associated with *Moraxella* spp. were recovered from the alimentary canal excretions. This bacterium is a polymorphous gram-negative opportunistic pathogen of both humans and cattle and is known to cause conjunctivitis in both animals. It also causes ear, nose and throat infections and is known to be transmitted by flies (Ala'Aldeen 2007).

Although many different types of bacterial taxa were recovered from the extracts of GWSS it is important to recall that these sequences are small relative to even the whole 16S gene. While these 500 bp sequences are sufficient to identify bacteria to the order and perhaps even family level, their predictive ability can be less absolute at the genus and species level. However, this data can be used to design primers to "walk" down the gene and may be more adept at resolving the species level identifications. In addition, because it is estimated that less that 10% of all bacteria have ever been fully identified this study has shown that many novel genera may be associated with the GWSS microbiome. Further study of the microflora of GWSS will be needed to identify possible targets of paratransgenesis or obligate symbiont knockdown.

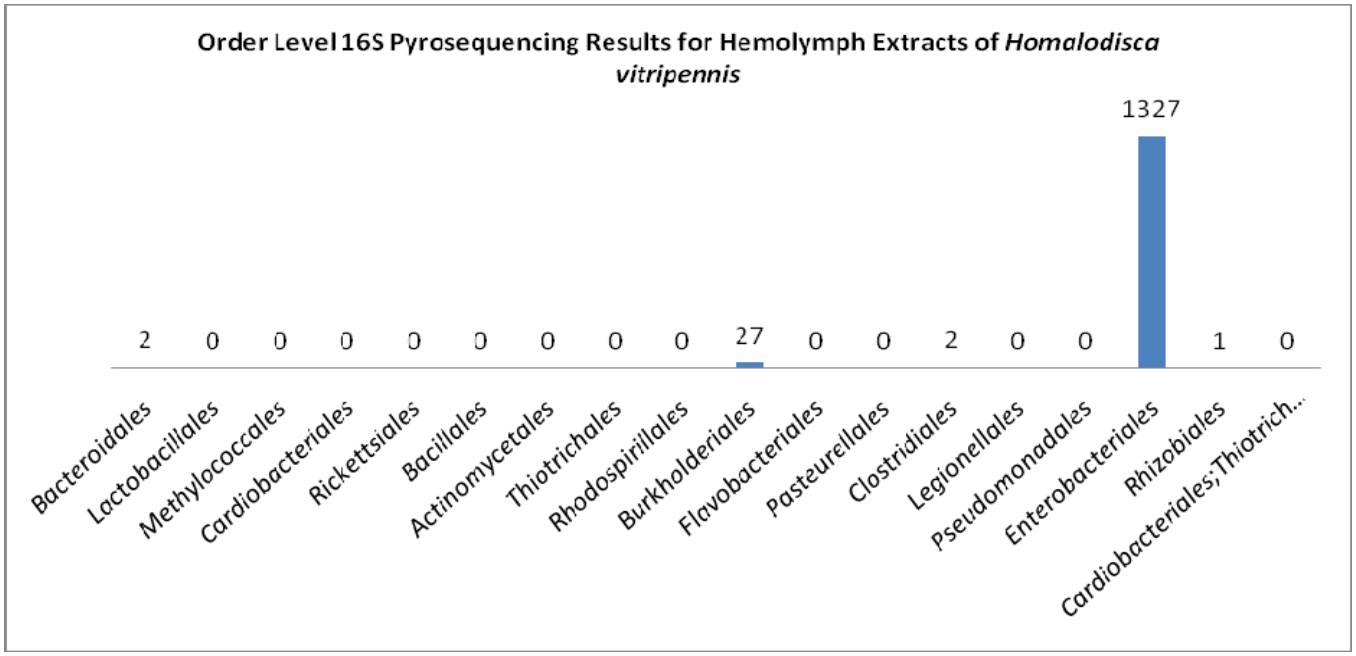

Figure 1. Order level sequencing results for the hemolymph of the GWSS. Larger numbers of sequences related to Enterobacteriales were recovered as well as Bacteroidales and Burkholderiales.

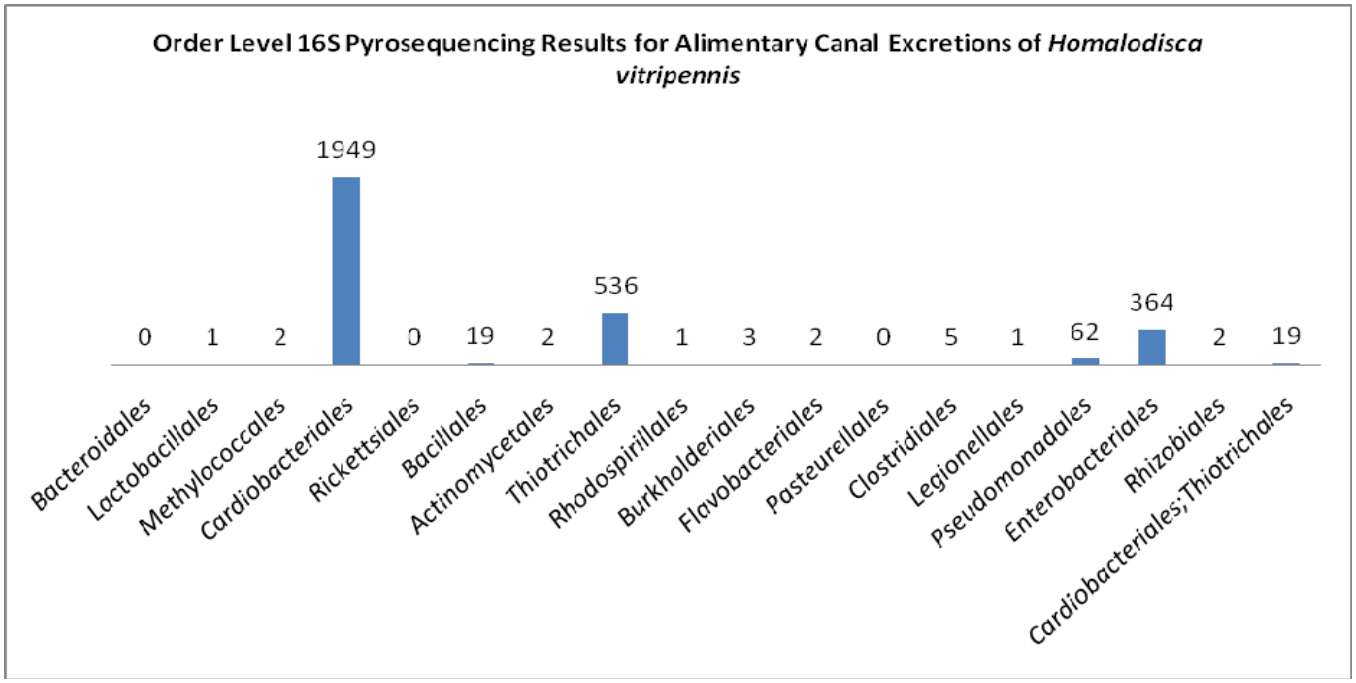

Figure 2. Order level sequencing results for the alimentary canal excretions of the GWSS. Sequences related to Cardiobacteriales, Thiotrichales, Enterobacteriales and others were recovered.

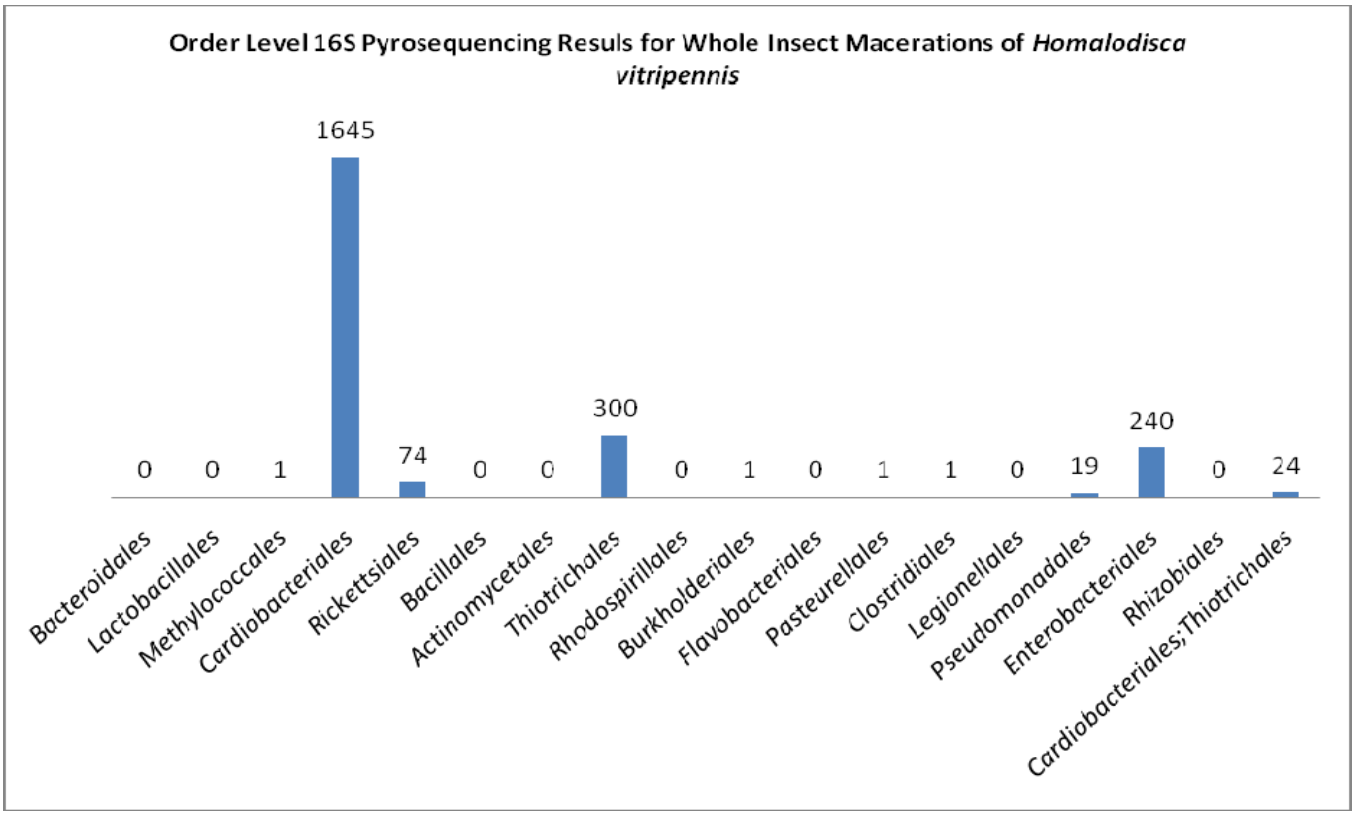

Figure 3. Order level sequencing results for the whole insect macerations of the GWSS. Sequences related to Cardiobacteriales, Thiotrichales, Enterobacteriales and others were recovered.

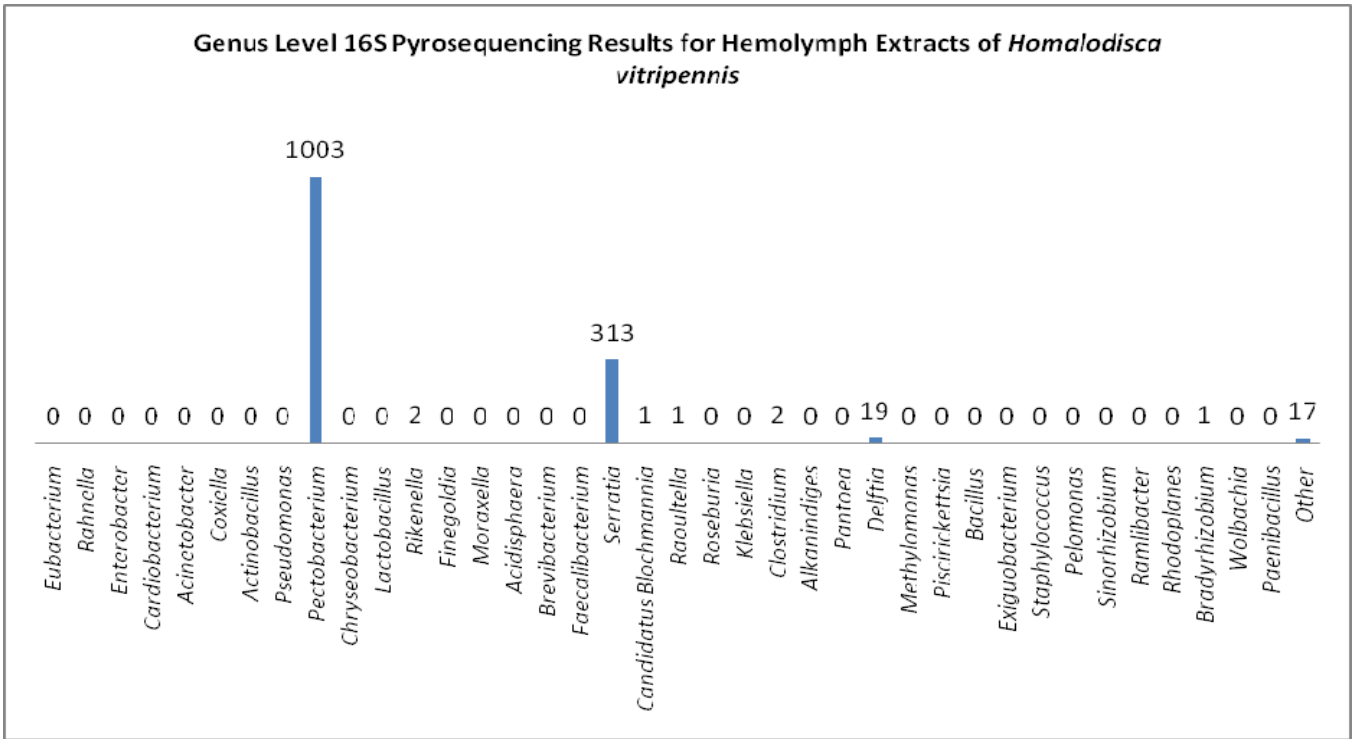

Figure 4. Genus level sequencing results for the hemolymph of the GWSS. Large numbers of *Pectobacterium* and *Serratia* were recovered. Some *Delftia* (formerly *Pseudomonas*) and other non-specific identifications were made. Note that no sequences from *Wolbachia*, an intracellular symbiont, were recovered.

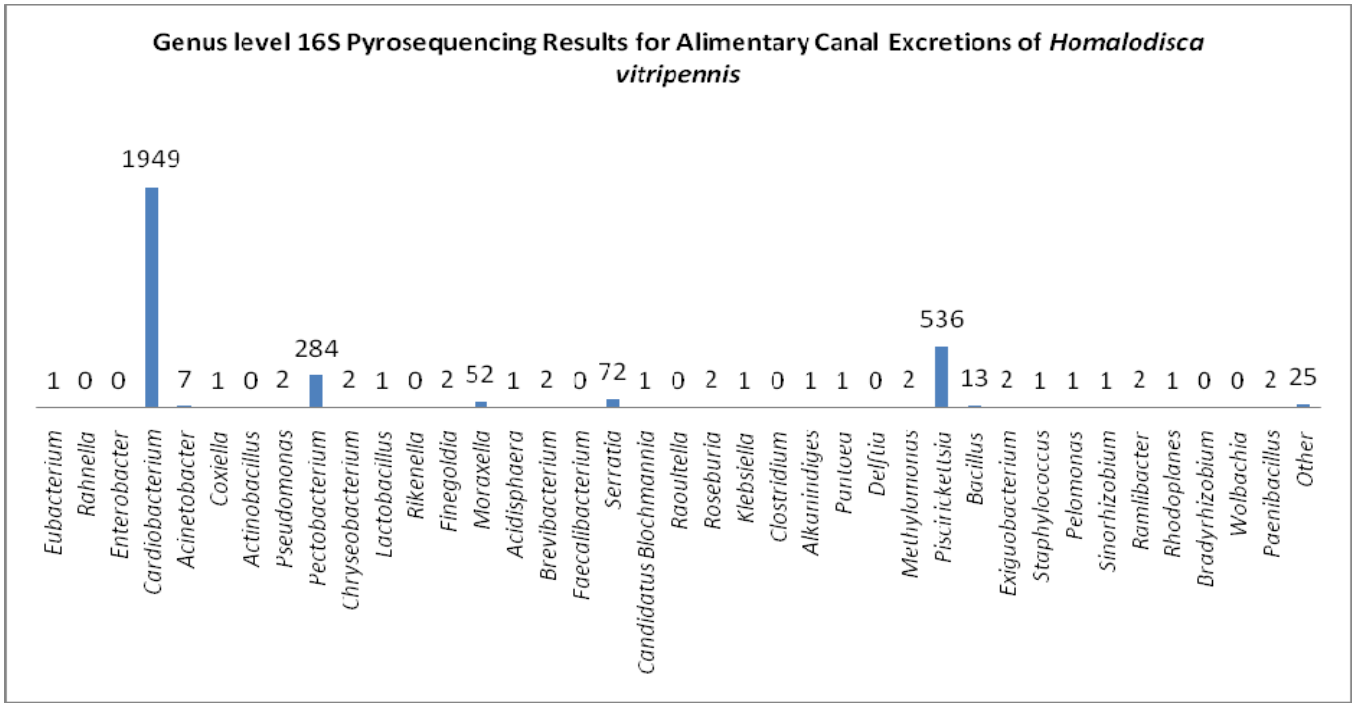

Figure 5. Genus level sequencing results for alimentary canal excretions of the GWSS. Many sequences of *Cardiobacterium*, *Pectobacterium, Piscirickettsia* and *Serratia* were recovered. Other non-specific identifications were made. Note that no sequences from *Wolbachia*, an intracellular symbiont, were recovered.

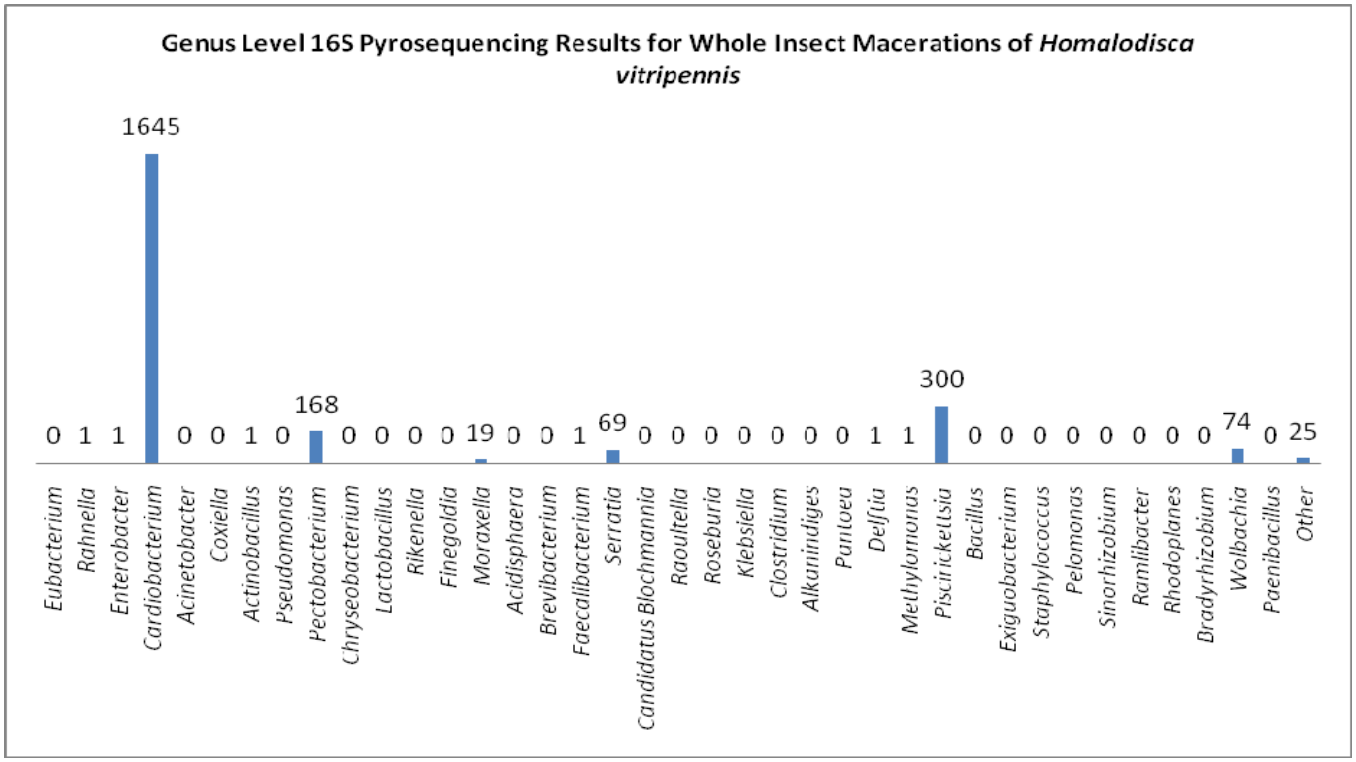

Figure 6. Genus level sequencing results for whole insect macerations of the GWSS. Many sequences of *Cardiobacterium*, *Pectobacterium, Piscirickettsia* and *Serratia* were recovered. Other non-specific identifications were made. Note that sequences from *Wolbachia*, an intracellular symbiont, were only recovered from whole tissue extracts.

CONCLUSIONS

Studies on the tsetse fly (*Glossina* spp.) have shown that transinfection by a genetically transformed strain of endosymbiont and expression of foreign gene products can be used to block transmission of a disease causing agent (Aksoy et al 2008). Paratransgenesis in the glassy-winged sharpshooter has not yet been extensively studied but one trial (Bextine et al 2004) did succeed in delivering *Alcaligenes xylosoxidans denitrificans*, expressing a red fluorescent protein to GWSS using a novel

feeding strategy. The bacterium was able to occupy the same area in the foregut normally associated with *Xf* infection. Using this system as a template and the results from the 16S pyrosequencing, we can now pursue multiple avenues of paratransgenesis.

The presence of *Delftia* sp exclusively in the hemolymph of GWSS provides an exciting possibility for this bacterium as a potential tool. A study by Wang et al (2008) showed that a new formulation of media was successful in culturing *Delftia tsuruhatensis* to a level 4.7 times higher than with un-optimized media. Other culture media are also available.

REFERENCES CITED

Ala'Aldeen, D.A.A. 2007. "Neisseria and moraxella". In Greenwood, David; Slack, Richard; Peitherer

Aksoy, S., B. Weiss, G. Attardo. 2008. Paratransgenesis applied for control of tsetse transmitted sleeping sickness. *Advances in Experimental Medicine and Biology*. 627:35-48

Barer, Mike (Eds.**),** Medical Microbiology (17th ed.), p. 258. Elsevier. ISBN 978-0-443-10209-7

Bextine, B., C. Lauzon, S. Potter, D. Lampe, T.A. Miller. 2004. Delivery of a Genetically Marked Alcaligenes sp. to the Glassy-Winged Sharpshooter for Use in a Paratransgenic Control Strategy. Current Microbiology. 48,327–331.

Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms. *Interscience*, New York, N.Y.

Chan, Y.C., H.P. Wu, D.Y. Chuang. 2009. Extracellular secretion of Carocin S1 in Pectobacterium carotovorum subsp. carotovorum occurs via the type III secretion system integral to the bacterial flagellum. *BMC Microbiology*. 9:181.

Curley, C.M., E.L. Brodie, M.G. Lechner and A H. Purcell. 2007. Exploration for Facultative Endosymbionts of Glassy-Winged Sharpshooter (Hemiptera: Cicadellidae). *Annals of the Entomological Society of America* 100 (3): 345-349.

Hai Xu, J. Davies and V. Miao. 2007. Molecular Characterization of Class 3 Integrons from Delftia spp. *Journal of Bacteriology*. 189, 17: 6276–6283

Hoddle, M.S., S.V. Triapitsyn, D.J.W. Morgan. 2003. Distribution and plant association records for *Homalodisca coagulata* (Hemiptera: Cicadellidae) in Florida. *Florida Entomologist*. 86, 89–91.

Lacava, P.T., J. Parker, F.D. Andreote, F. Dini-Andreote, J.L. Ramirez and T.A. Miller. 2007. Analysis of the bacterial community in glassy-winged sharpshooter heads. *Entomological Research* 37: 261–266.

Mavingui, P., V.T. Van, E. Labeyrie, E. Rancès, F. Vavre and P. Simonet. 2005. Efficient Procedure for Purification of Obligate Intracellular Wolbachia pipientis and Representative Amplification of Its Genome by Multiple-Displacement Amplification. *Applied and Environmental Microbiology*. 71, 11: 6910-6917

Moran, N.A., P. Tran and N.M. Gerardo. 2005. Symbiosis and Insect Diversification: an Ancient Symbiont of Sap-Feeding Insects from the Bacterial Phylum *Bacteroidetes*. *American Society for Microbiology*. 71, 12: 8802–8810.

Neelakanteshwar, K.P., R. Kundapur, Y.S. Shouche and T.B. Karegoudar. 2006. Degradation of a Plasticizer, di-n-Butylphthalate by Delftia sp. TBKNP-05. *Current Microbiology*. 52: 225–230

Turner, W.F., N. Pollard. 1959. Life histories and behavior of five insect vectors of phony peach disease. Technical Bulletin 1188, US Department of Agriculture, 28pp.

Wu D, Daugherty SC, Van Aken SE, Pai GH, Watkins KL, et al. 2006. Metabolic Complementarity and Genomics of the Dual Bacterial Symbiosis of Sharpshooters. *PLoS Biol* 4 (6): e188. doi:10.1371/journal.pbio.0040188

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IDENTIFICATION AND WHOLE EXTRACTION OF *HOMOLADISCA COAGULATA-VIRUS01 (HoCV-01* FROM TEXAS GLASSY-WINGED SHARPSHOOTER POPULATIONS

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ABSTRACT

The glassy-winged sharpshooter (GWSS) is an invasive pest and important vector of *Xylella fastidiosa (Xf)*, a xylem-limiting bacteria that causes Pierce's disease (PD) in grapes as well as other agricultural diseases. The primary method of managing the spread of *Xf* is controlling its insect vector populations. Methods such as chemical control are not target specific and lead to problems such as residue contamination, injury to non-target organisms, and insecticide resistance. Identifying agents that can impact GWSS populations is the goal of a biological control strategy. In this study, we have identified and extracted whole GWSS *Virus 01 (HoCV-01)* from populations of GWSS collected in Texas. *HoCV-01* is a novel virus that harbors pathogenic potential with regard to GWSS. Future plans for *HoCV-01* include reintroduction into GWSS populations through feeding. Increased amounts of *HoCV-01* ingestion may lead to weakened populations of GWSS that are more susceptible to control methods such as insecticides. This would decrease the amount of insecticide needed to produce a desired mortality rate in insect populations.

LAYPERSON SUMMARY

The glassy-winged sharpshooter (GWSS) is the most economically important insect with respect to the spread of *Xylella fastidiosa (Xf)*, the causal agent of Pierce's disease (PD). Therefore control of this insect is of paramount importance to the management of the disease. While insecticides have been used successfully to reduce the economic impact of this disease system, alternate methods of population insect control are needed to continue management in the future. Biological control offers alternatives to chemical control that can be effective in negatively impacting insect population without harmful environmental effects or concern for insecticide resistance. In this work, we molecularly describe a virus that shows promise as a tool for biological control. While this virus does not cause significant acute mortality, it may reduce the fitness of insects to a point where other control methods would be more effective. We suggest that viral infection will make insects more sensitive to insecticide treatment, resulting in lower LD50 rates for achievement of significant control. This means that lower levels of insecticide can be used effectively.

INTRODUCTION

The glassy-winged sharpshooter (GWSS) is the major vector of *Xylella fastidiosa (Xf)* Wells in the southern USA (Adlerz 1980; Blua et al., 1999). The plant pathogenic bacterium, *Xf*, has caused economic losses to several agricultural industries in North America and is associated with many plant diseases such as Pierce's disease (PD), and oleander leaf scorch. PD of grapevine has become a well recognized *Xylella*-related disease; the vector profile is well known and the epidemiology of the disease is well documented (Hopkins et al., 2002). The introduction of GWSS into new areas is directly related to increased occurrence of PD in vineyards (Perring et al., 2001). Therefore, the management of PD depends heavily on the ability to control its vectors, especially GWSS.

Methods of vector manipulation such as chemical control with the use of insecticides are not target specific and lead to problems such as residue contamination, injury to non-target organisms, and insecticide resistance. The search for more benevolent pest management strategies has led to the use of biocontrol agents such as fungi and parasitoids. However, by utilizing viruses that currently reside in GWSS populations,a viral bio-control that is even more precise may be developed (Hunnicutt et al., 2006).

HoCV-01 is a member of the genus *Cripavirus* and family *Dicistroviridae (*Hunnicutt et al., 2006). It is a novel virus that harbors pathogenic potential with regard to GWSS. The focus of this study was the identification and extraction of whole *HoCV-01* found in populations of GWSS collected in Texas. Once identification was complete, the genome was sequenced and checked for variation which may produce an increase in virulence. Following sequencing, whole *HoCV-01* was extracted in order to reintroduce it into GWSS populations.

OBJECTIVES

1. Identify *HoCV-01* in populations of GWSS collected in Texas.
2. Sequence viral capsid protein and check for variation between strain found in Texas and strain found in California.
3. Extract and purify whole *HoCV-01* with intent to reintroduce into uninfected populations of GWSS.

MATERIALS AND METHODS

RNA Extraction. GWSS bodies were collected in microcentrifuge tubes and homogenized. GWSS RNA was separated from the solution and purified using a Qiagen RNeasy kit (Qiagen™, Germantown, MD).

RT-PCR & Gel Electrophoresis. Each 1µL GWSS RNA sample was combined with 10µL 2X Reaction Mix (Invitrogen Molecular Probes™, Eugene, OR), 0.4µL forward primer, 0.4µL reverse primer, 0.4µL Platinum® Taq DNA Polymerase (Invitrogen Molecular Probes™, Eugene, OR), and 8µL DEPC H_2O in solution. Samples were then subjected to a Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) using an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). 2µL of each GWSS DNA sample was subjected to gel electrophoresis using 2µL ladder, 2µL loading dye per sample, and a 1% agarose gel containing 100mL TAE buffer and 1g agarose gel. Gels were subjected to 100V and 400A for 50 min. and checked under Ultraviolet light in a Bio Doc-It Imaging System (Cole-Parmer™, Hanwell, London).

Sequencing PCR & Ethanol Precipitation. Sequencing was done on sight in the Bextine Molecular Biology Laboratory at The University of Texas at Tyler. An amount of 2µL of each GWSS DNA sample was combined with 2µL Nano-pure H_2O, 2µL primer (only the forward or the reverse primer were used in this step), 4µL GenomeLab™ DTCS Quick Start Mix (GenomeLab™, Fullerton, CA), and taken through a Sequencing Polymerase Chain Reaction, or Sequencing PCR in an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). Each sample was then combined with 2µL 3M NaOAc, 2µL 100nM EDTA, 1µL 20mg/mL Glycogen, vortexed thoroughly, and subjected to an ethanol precipitation. During the ethanol precipitation, sample DNA was purified for sequencing with separate washes of ice cold 95% and 70% ethanol. The resulting pellet of purified DNA was mixed with 40µL Sample Loading Solution, vortexed, and transferred to a sequencing plate.

DNA Sequencing. DNA samples were sequenced using a CEQ 8000 Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA). Prior to loading the samples into the sequencer, each sample was combined with one drop of Mineral Oil. Data was analyzed using Bioedit® Sequence Alignment Editor (Ibis Biosciences, Carlsbad, CA).

Whole Virus Extraction. Infected GWSS bodies were placed in a mortar and pestle and homogenized in 100mL of phosphate buffer containing 0.02mg DETCA. The homogenate was then transferred to 50mL centrifuge tubes and centrifuged at 1600rpm for 20 minutes in an Eppendorf 5804R Centrifuge (Eppendorf , Hamburg, Germany). The resulting supernatant was split into two ultra-centrifuge tubes, combined with more phosphate buffer with DETCA, vortexed, and ultra-centrifuged at 22,000rpm for 16 hours in a Sorvall® RC-5B Refrigerated Superspeed Centrifuge (DuPont Instruments, Wilmington, DE). Following ultra-centrifugation, the supernatant was discarded, and the pellet was dissolved with 5mL phosphate buffer with 0.4% Na-deoxycholic acid and 4% Brij 52. The resulting solution was centrifuged and 1600rpm for 15 minutes, passed through a 0.45µm filter, and collected into large Eppindorf tubes. The unrefined *HoCV-01* solution was placed in a dialysis membrane, placed in a large beaker containing a stir-bar and ddH_20, and placed in a refrigerator at 4°C. The ddH_20 was changed out ever five-six hours until a white precipitate could be seen in the dialysis membrane. The purified *HoCV-01* solution was collected into micro-centrifuge tubes and stored at -80°C.

RESULTS AND DISCUSSION

HoCV-01 was detected in GWSS populations collected in Texas. Sequence comparison of the Texas strain of *HoCV-01* against the sequenced California strain (Hunnicutt et al., 2006) shows some variation. The percent similarity between the strains is **98.8%.**

Base pair 828, Cytosine in the California strain, is a Thymine in the consensus strain (**Figure 1**). This changes the amino acid translation from Serine (polar side chain) into Leucine (non-polar side chain). Also, due to a Guanine insertion in the consensus strain at base pair 904 (possibly a deletion in the California strain), variation downstream in the amino acid chain was observed (**Figure 1**).

The presence of variation between the Texas *HoCV-01* sequence and the California *HoCV-01* sequence is a possible indication that the Texas strain may exhibit increased virulence. The Guanine insertion at base pair 904 caused variation in all downstream amino acid translation. This could lead to changes in protein folding and ultimately changes in protein function. Altered protein functions may cause an increase in virulence in the Texas *HoCV-01* strain.

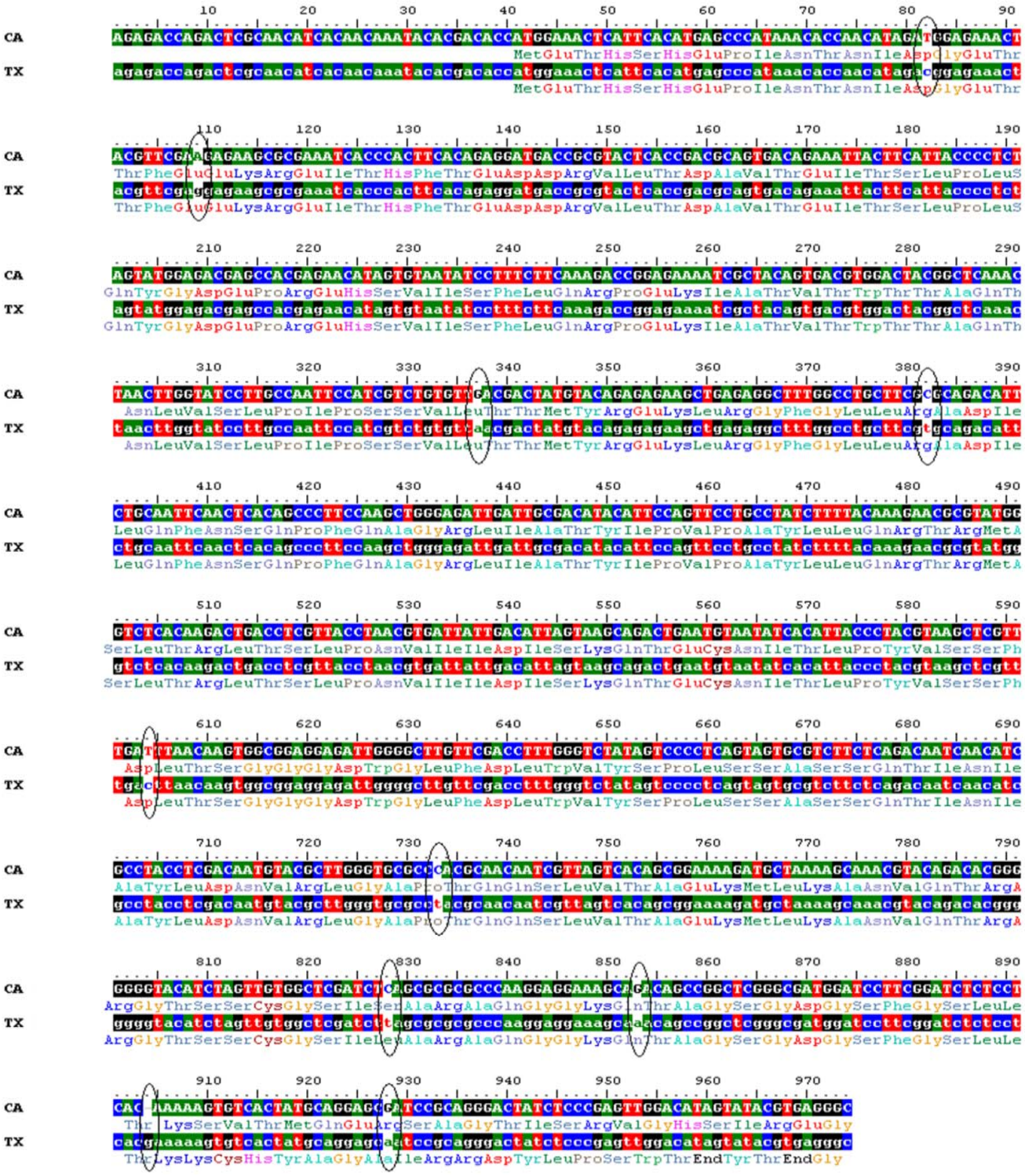

Figure 1. Sequence comparison with protein translation of the California *HoCV-01* strain and the Texas HoCV-1 strain. Note the circled areas which indicate variation between the two strains.

CONCLUSIONS

The presence of *HoCV-01* in populations of GWSS collected in Texas is crucial in developing an ideal viral bio-control and pest management strategy. The possible increase in virulence in the Texas strain is also an indication of the pathogenic potential of *HoCV-01*. This novel virus has been extracted and purified. In a following study, purified *HoCV-01* will be reintroduced to a population of uninfected GWSS in order to determine increases in population weakness or mortality that may result. The results of this experiment are crucial in further understanding the insect vector, GWSS. The management of PD depends heavily upon the ability to control its insect vectors.

REFERENCES CITED

Adlerz, W. C. 1980. Ecological observations on two leafhoppers that transmit the Pierce's disease bacterium. Proc. Fla. State Hortic. Soc. 93:115-120.

Blua, M. J., Phillips, P. A., and Redak, R. A. 1999. A new sharpshooter threatens both crops and ornamentals. Calif. Agric. 53 (2):22-25.

Hopkins, D. L., and Purcell, A. H. 2002. *Xylella fastidiosa*: Cause of Pierce's Disease of Grapevines and Other Emergent Diseases. Amer. Phytopath. Soc. 86 (10):1056-1064.

Hunnicutt, L. E., Hunter, W. B., Cave, R. D., Powell, C. A., and Mozoruk, J. J. 2006. Genome sequence and molecular characterization of *Homalodisca coagulata virus-1*, a novel virus discovered in the glassy-winged sharpshooter (Hemiptera: Cicadellidae). Virology. 350 (1): 67-78.

Perring, T. M., C. A. Farrar, and M. J. Blua. 2001. Glassy-winged sharpshooter host impacts Pierce's disease in Temecula Valley vineyards. Calif. Agric. 55: 13-18.

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PHYLOGENETIC ANALYSIS OF HEAT SHOCK PROTEINS IN THE GLASSY-WINGED SHARPSHOOTER

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Reporting Period: The results reported here are from work conducted January 2009 to December 2009.

ABSTRACT
The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis* (Germar); Hemiptera: Cicadellidae) is the major vector of *Xylella fastidiosa (Xf)*, the causal agent of Pierce's disease (PD) of grapes. As genomic information becomes available more research on leafhopper stress responses are possible. Due to the importance of the GWSS in transmission and spread of *Xf*, a cDNA library was constructed from adult and fifth-instars GWSS, resulting in 5,906 expressed sequence tags (ESTs). After quality scoring, 4,445 sequences underwent assembly which produced a set of 2,123 sequences that putatively represented distinct transcripts. BLASTX analysis identified four significant homology matches to heat shock proteins, (HSP) which are the focus of this study. The overall importance and function of HSPs lie in their ability to maintain protein integrity and activity during stressful conditions, such as extreme heat, cold, drought, or other stresses. Phylogenetic analyses using these four HSP sequences provided further support of transcript by the identification of specific motifs. This study shows that highly conserved genes like HSPs are a viable alternative to ribosomal DNA in elucidating phylogenetic relationships.

LAYPERSON SUMMARY
In this study, we generated and analyzed a cDNA library, which is a representation of all the protein coding genes in an organism, of the insect pest glassy-winged sharpshooter (GWSS). We isolated four incomplete sequences from a large family of proteins called heat shock proteins (HSP). Although they are called HSP, these proteins actually perform many activities in the cell that allow for GWSS to survive stresses like extreme temperatures, pesticides, and even viral infection. This study compares the HSP sequences from GWSS to those of other insect species to help describe the relationship and history of GWSS to find ways to track changes in GWSS territory and life history.

INTRODUCTION
Organisms respond to heat shock or other environmental stress by inducing the synthesis of proteins some of which are known as heat shock proteins (HSP) (Lindquist 1986, Sorenson 2003). Infections, temperature changes, inflammation, toxins, hypoxia, starvation and even exercise can result in increased production of heat shock proteins (Sorenson 2003). HSP aid in folding, targeting and tracking of nascent proteins, promote transcription, are involved in cellular division and can be up regulated via cell signaling in addition to environmental stimuli (Feder and Hofmann 1999).

Small heat shock proteins (sHSP) have an approximate molecular weight of less than 30kDa and are molecular chaperones, maintaining proper protein structure by blocking aggregation of denaturing proteins, aiding nascent protein folding and assisting construction of quaternary structure (Fu and Chang 2004, Gu et al. 2002, Bova et al. 2002, Sobott 2002).

Among the HSP families is a group of well-conserved proteins with an approximate molecular weight of 70 kDa, known as the HSP70 family. Most species have several proteins belonging to this family. Some of these members are only expressed under stress conditions (strictly inducible), while some are present in cells under normal growth conditions (Craig 1989) and are not heat-inducible (Pelham 1986), and are known as heat shock cognates (HSC). In eukaryotes, HSP70 can work with sHSP to restore functionality to heat-denatured proteins (Lee and Vierling 2000) or co-chaperone with HSP40 to fold nascent proteins into proper tertiary structure by temporarily binding to hydrophobic domains until sequence translation is complete (Douglas et al. 1994).

The 90 kDa heat shock proteins (HSP90) is one of the most prolific proteins in eukaryotic cells, constituting 12% of cellular proteins under baseline conditions (Sreedhar 2004). Their functions and morphological evolution have been extensively studied and include signal transduction, protein folding and degradation of denatured proteins (Nadeau 1993, Jakob 1994). Increased functionality of HSP90 is acquired when associated with its co-chaperones, playing an important role in the folding of newly synthesized proteins. Apart from its co-chaperones, HSP90 binds to an array of substrate proteins, where the necessary co-chaperones varies and depends on the actual substrate (Jakob 1995). Understanding heat shock proteins in insects, especially leafhoppers, will provide insights into the biological adaptive elasticity of these important agricultural pests to stressors such as insecticides, parasitization, and temperature.

The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis* (Germar); Hemiptera: Cicadellidae), is an insect pest that occurs throughout most of the southern USA and is endemic to most regions of Texas (Young 1958; Turner and Pollard 1959). Without naturally occurring forms of biological control, GWSS have established populations in new areas and have

negatively affected the yields of the grape industry (de Leon et al. 2004). GWSS is a voraciously-feeding, xylem-limited pest that has been reported to feed on host plants from at least 35 families, including both woody and herbaceous types (Hoddle et al. 2003), and can impact the plant's health directly by depriving the plant of nutrients and damaging the xylem sufficiently to preclude vascular flow. However, indirect plant damage occurs during feeding and subsequent transmission of the xylem-limited bacterium *Xylella fastidiosa (Xf)* Wells (Xanthomonadales: Xanthomonadaceae). The invasion of GWSS into grape growing regions of California, namely the Temecula valley, produced an enormous risk to the California wine and table grape industry by spreading the phytopathogen *Xf,* the causative agent of Pierce's disease (PD) (Purcell 1997, de Leon et al. 2004). Additionally, many other economically important plants including citrus, almond and oleander are affected by separate strains of *Xf* resulting in a multitude of plant diseases including citrus variegated chlorosis (Chang et al. 1993; Pooler and Hartung 1995), almond leaf scorch (Mircetich et al. 1976) and oleander leaf scorch (Purcell 1999).

A search of the National Center for Biotechnology Information (NCBI) for GWSS genes or protein sequences revealed less than 25 complete, non-mitochondrial genes or complete proteins. Although the complete mitochondrial sequence of GWSS has been described (Genbank AY875213), the genomic DNA sequence is incomplete. Over 20,000 Expressed Sequence Tags (ESTs) from GWSS have been submitted to NCBI; however, many of these EST's are duplicates and this study is an initial step in examining the potential use of this information by examining the utility of these heat shock proteins to describe the phylogeny of leafhoppers in relation to other insects. Further management approaches have been proposed to disrupt HSP in insects as a means to suppress leafhopper populations.

OBJECTIVES

1. Identify the phylogenic relationship of important leafhopper species using heat shock protein sequences.
2. Develop a methodology to distinguish between populations of GWSS.

RESULTS AND DISCUSSION

Mining of the 5,906 cDNA clones produced from cDNA library constructed from 140 adult and fifth-instar GWSS using Stratagene's ZAP-cDNA Synthesis Kit (Stratagene, La Jolla, CA, USA) resulted in 4,445 high-quality (i.e., ≥200 bases with a TraceTuner™ score of 20 or better) GWSS ESTs sequenced by ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence alignment of these ESTs resulted in a Unigene set of 2,123 total assembled sequences, at Phred 20 score, 40bp overlap, 100 bp minimum length, using Sequencher™ 8.0, (Gene Codes Corp, Ann Arbor, MI, 48108). Translated proteins were analyzed with National Center for Biotechnology Information's (NCBI) BLAST*p*, Pfam (www.pfam.org), InterProScan (www.ebi.ac.uk) and Expert Protein Analysis System (www.expasy.org). Four partial protein sequences were analyzed for phylogenetic relationships with homologous HSP sequences. A BLAST*p* and BLAST*n* analysis showed that WHWTC-contig[1627] and WHWTC-contig[1325] showed significant homology with the sHSP, while WHHC-contig[1333] displayed homology with HSP70, and WHWTC-contig[1285] was homologous with HSP90 (**Table 1**). A homology search conducted in the Pfam database identified protein sequences with their respective HSP families (**Table 2**). A functional analysis and homology search using PantherDB annotated and classified the sequences as belonging to the HSP superfamily (data not shown).

Table 1. Protein sequence similarities from GWSS contigs. Nucleotide matches (accesion|protein description organism) and e-values for query contigs using National Center for Biotechnical Information (NCBI)'s BLAST*x*.

GWSSclones	**Descriptor**	**E-Value**
WHWTC-Contig[1627] 694 bases	gb|ABC84494.1| heat shock protein 20.7 *Locusta migratoria*	3.00E-43
WHWTC-Contig[1325] 954 bases	gb|ACH85196.1|heat shock protein 20 *Bemisia tabaci*	2.00E-44
WHHC-Contig[1333] 1047 bases	gb|AAZ17399.2| 70 kDa heat shock protein *Bemisia tabaci*	2.00E-149
WHWTC-Contig[1285] 1144 bases	gb|AAZ17403.1| 90 kDa heat shock protein *Bemisia tabaci*	6.00E-179

Table 2. Hidden Markov models (HMM) homology search of *in silico*-translated protein sequences using Pfam protein database (www.pfam.org) with protein family description, Pfam identification, sequence coverage and corresponding e-value.

Contig Number	Description	Pfam Family ID	Sequence		HMM		E-value
			Start	End	From	To	
WHWTC-Contig[1627]	Hsp20/alpha crystallin family	PF00011	86	182	1	109	1.50E-40
WHWTC-Contig[1325]	Hsp20/alpha crystallin family	PF00011	63	159	1	109	2.30E-38
WHHC-Contig[1333]	Hsp70 protein	PF00012	1	325	291	619	2.20E-201
WHWTC-Contig[1285]	Hsp90 protein	PF00183	3	380	101	489	0

The heat shock proteins (HSPs) from GWSS had homology to the HSP from other insects, and grouped most closely with other Hemiptera when subjected to phylogenetic analysis. Phylogenetic trees illustrated accurate grouping of taxa into clades relative to known HSP from closely related Hemipteran species (**Figures 1-3**). The clades were separated according to taxonomic Order. Two small heat shock protein sequences (sHSP) from GWSS grouped with another sharpshooter *Graphocephala atropunctata* sHSP (**Figure 1**). The HSP70 sequence from GWSS grouped with two HSP70 sequences from the pea aphid (*Acyrthosiphon pisum*) (**Figure 2**). Finally, the HSP90 sequence from GWSS grouped with three sequences from the pea aphid (*A. pisum*) (**Figure 3**).

These phylogenetic analyses corroborate evidence from Pfam and PantherDB protein databases that describe the GWSS partial protein sequences as HSP. Additionally, the phylogenetic trees created using these protein sequence comparisons show that HSP can be used to determine phylogenetic and cladistical associations.

Heat shock proteins, HSPs have a variety of functions within the cell including the prevention of protein aggregation and denaturation due to heat and are well conserved across all taxa, and are present in every species analyzed (Feder and Hofmann 1999, Sorensen 2003). HSP families are organized via their level of expression in the cell (i.e. inducible or constitutive expression) as well as the complexes formed by the HSP; however, the greatest organization criteria is the molecular weight of the HSP which include families of 20kDa, 40kDa, 60kDa, and 90kDa proteins (Gething 1997). Finally, conserved domains exist in these families, including alpha-crystalline structure in sHSP, an N-terminal pentapeptide sequence in HSP70, and a highly conserved N-terminal domain in HSP90. The HSP sequences collected from GWSS contain these conserved domains permitting significant *in silico* comparisons (data not shown).

Phylogenetic analysis and alignment searches of the four HSP sequences were confounded by the overwhelming number of HSP sequences isoforms submitted to NCBI. However, with careful consideration paid to HSP isoforms, phylogenetic comparisons showed accurate clades of GWSS HSP with those of other closely related insect taxa (**Figures 1-3**). Phylogenetic trees formed on HSP comparison verified other phylogenetic analyses based on mitochondrial DNA. Although many ESTs in this study were found to be HSP homologues, there remain many as yet unanalyzed HSP in the GWSS genome, including members of the small Heat Shock Proteins (sHSP), HSP60, and HSP70 families. Additionally, many members of the HSP90 family and its co-chaperones have yet to be sequenced. The need for more in depth sequencing of GWSS is evident by the paucity of HSPs currently identified in the GWSS genomic database. In *Drosophila melanogaster*, whose genome is completely sequenced, over 200 HSPs have been identified and submitted to the National Center for Biotechnology Information (NCBI). GWSS has a predicted genome size of ~1.24 pg (Hunter, unpublished), similar to the haploid male Whitefly, *Bemisia argentifolii* at ~1.1pg (Leshkowitz et al. 2006), three times the size of the Asian citrus psyllid ~0.35pg (Hunter et al., 2009) and roughly five times the size of the fruitfly *D. melanogaster*, which is ~0.18pg (Brown et al. 2005). Thus, we suspect that sharpshooters will have a number of HSPs that would approximate the number in other insect genomes.

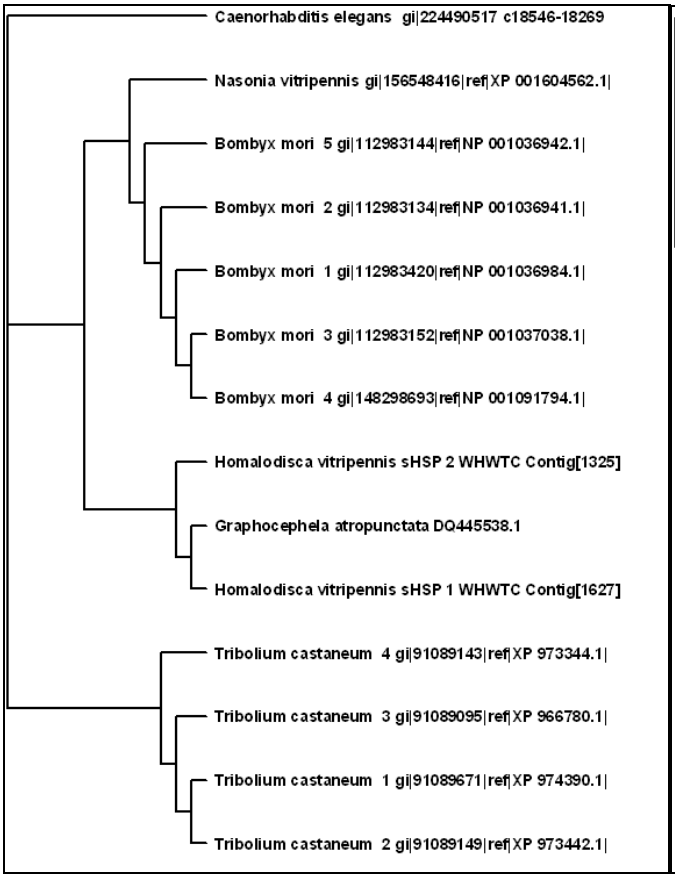

Figure 1. Cladogram of small heat shock proteins constructed using Glassy-winged sharpshooter sequences WHWTC-Contig[1627] and WHWTC-Contig[1325]. Subject sequences were analyzed using NCBI BLAST*p* search and aligned using T-Coffee multiple alignment tool (www.tcoffee.org) and visualized using Treeview v1.6.6. (Organism name|accession number| reference number).

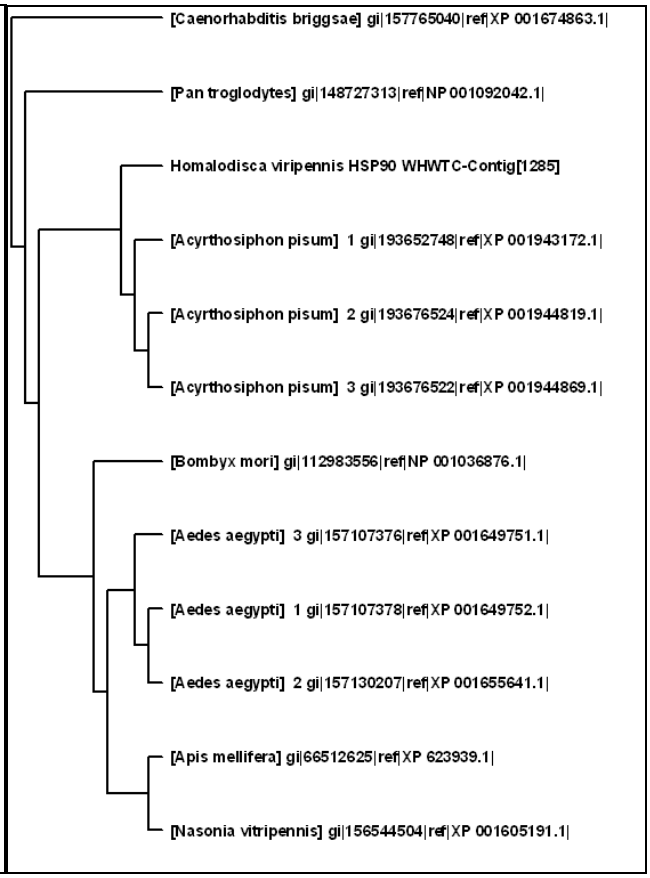

Figure 2. Cladogram of heat shock 70 proteins constructed using sequence WHHC-Contig[1333]. Subject sequences were analyzed NCBI BLAST*p* search and aligned using ClustalW2 multiple alignment tool (www.ebi.ac.uk/Tools/clustalw2/index.html) and visualized using Treeview v1.6.6. (Organism name|accession number| reference number).

Systematic biases can distort evidence via improper gene sampling. Therefore, it is necessary to limit the effects of these entanglements by analyzing multiple genes that undergo relatively uniform evolution. Ribosomal DNA is a useful molecule for examining phylogenetic relationships among many eukaryotes, primarily because no other molecule has been sequenced as extensively (Stechmann 2003). However, phylogenetic analysis using ribosomal DNA can cause artefactual groupings of unrelated genera that have undergone rapid rRNA evolution (Philippe and Adoutte 1998; Philippe et al. 2000). Previous studies have used HSPs to elucidate phylogenetic relationships in eukaryotes (Plesofsky-Vig 1992, Stechmann 2003). The ubiquitous and metropolitan prevalence of HSP allow for comparison of organisms as distantly related as that of bacteria, *Escherichia coli* and flies, *Drosophila melanogaster* (Lindquist 1986). Additionally, the importance of HSP in evolution and speciation has been well documented (Sorensen 2003). Finally, the difference between families of HSPs allows researchers many options in determining precision and resolution in describing phylogenetic relationships by utilizing the more conserved HSP90 domain or the more varied sHSP family to define relationships at any level of categorization, from kingdom to species (Feder and Hofmann 1999). One of the greatest determining factors in host range for an invasive species is stress tolerance; an attribute directly related to HSP expression (Feder and Hoffman 1999). As such, the sequence variation of heat shock proteins offer an excellent resource to apply in defining phylogenetic relationships, and also to aid in revealing pest species range.

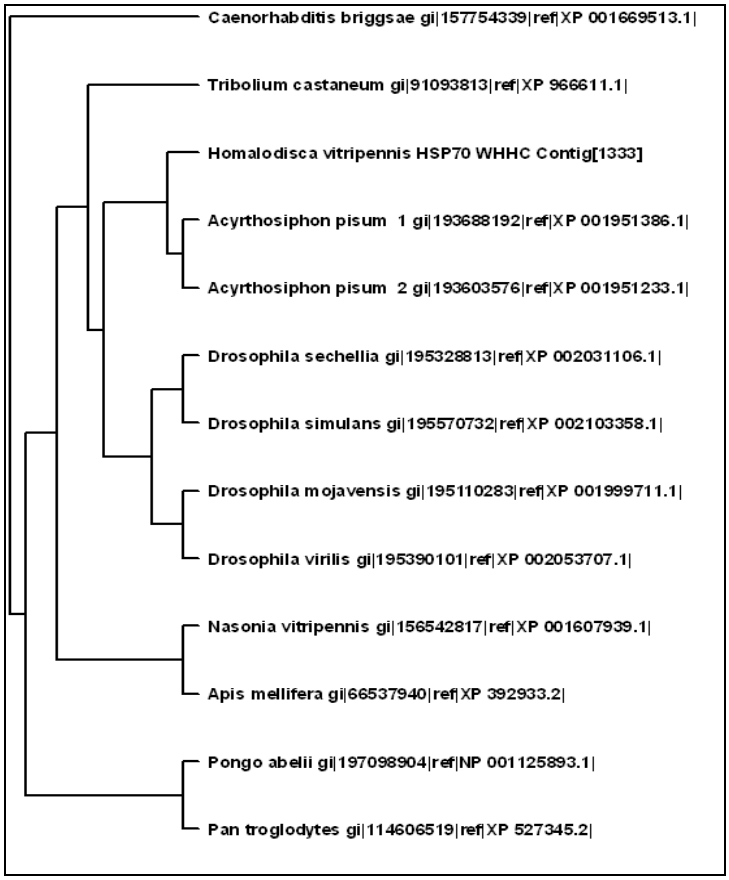

Figure 3. Cladogram heat shock 90 proteins constructed using sequence WHWTC-Contig[1285]. Subject sequences were analyzed using NCBI BLAST*p* search and aligned using ClustalW2 multiple alignment tool (www.ebi.ac.uk/Tools/ clustalw2/ index.html) and visualized using Treeview v1.6.6. (Organism name|accession number| reference number).

CONCLUSION

These results show that HSP can be used to accurately describe the phylogenetic history of GWSS, thus offering a novel target for molecular systematics. Additionally, this study is the first to describe any of the HSP sequences found inGWSS. We believe that understanding and sequencing heat shock protein encoding genes is an important step elucidating the underlying genetic determinants of pest species range and stress tolerance of GWSS.

REFERENCES CITED

Bateman A, Coin L, Durbin R, Finn RD, Hollich V, Griffiths Jones S, Khanna A, Marshall M, Moxon S, Sonnhammer E L, Studholme DJ, Yeats C, Eddy SR. (2004) The Pfam protein families database. *Nucl. Acids Res.* 32: D138-D141.

Bova MP, Huang Q, Ding L, Horwitz J. (2002) Subunit exchange, conformational stability, and chaperonelike function of the small heat shock protein 16.5 from *Methanococcus jannaschii*. *J Biol Chem* 277: 38468–38475.

Bukau B, Horwich AL. (1998) The Hsp70 and Hsp60 chaperone machines. *Cell.* 92: 351-66.

Burdon RH. (1986) Heat shock and the heat shock proteins. *Biochem J*. 240: 313–324.

Craig EA. (1989) Essential roles of 70kDa heat inducible proteins. *Bioessays.* 11: 48-52.

Chang CJ, Garnier M, Zreik L, Rossetti V, Bove JM. (1993) Culture and serological detection of xylem-limiting bacterium causing citrus variegated chlorosis and its identification as a strain of *Xylella fastidiosa*. *Curr Microbiol* 27: 137-142.

Coudron TA, Brandt SL, Hunter WB. (2006) Molecular profiling of proteolytic and lectin transcripts in *Homalodisca vitripennis (*Hemiptera: Auchenorrhyncha: Cicadellidae) feeding on sunflower and cowpea. *Arch. of Insect Biochem. and Physiol*. 66: 76-88.

Cyr DM, Langer T, Douglas MG. (1994) DnaJ-like proteins: molecular chaperones and specific regulators of Hsp70, *Trends Biochem Sci*. 19: 176-181.

Feder ME, Hofmann GE. (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Ann Rev Physio.* 61: 243–282.

Flaherty KM, DeLuca-Flaherty C, McKay DB. (1990) Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein. *Nature*. 346 623-628.

Fu X, Chang Z (2004) Temperature-dependent subunit exchange and chaperone-like activities of Hsp16.3, a small heat shock protein from Mycobacterium tuberculosis. *Biochem Biophys Res Commun* 316: 291–299.
Gething MJ, ed. 1997. Guidebook to Molecular Chaperones and Protein-Folding Catalysts. Oxford, UK: Oxford Univ. Press.
Gu L, Abulimiti A, Li W, Chang Z (2002) Monodisperse HSP16.3 nonamer exhibits dynamic dissociation and reassociation, with the nonamer dissociation prerequisite for chaperone-like activity. *J Mol Biol* 319: 517–526.
Hoddle MS, Triapitsyn SV, Morgan DJW. (2003) Distribution and plant association records for *Homalodisca coagulata* (Hemiptera: Cicadellidae) in Florida. *Florida Entomol* 86: 89–91.
Hunter WB, Dowd SE, Katsar CS, Shatters RG Jr, McKenzie CL, Hall DG. (2009) Psyllid biology: expressed genes in adult Asian citrus psyllids, *Diaphorina citri* Kuwayama. *Open Entomol J*. 3: 18-29.
Jakob U, Buchner J. (1994) Assisting spontaneity: the role of Hsp90 and small Hsps as molecular chaperones. *Trends Biochem Sci.* 19: 205-211.
Jakob U, Lilie H, Meyer I, Buchner J. (1995) Transient interaction of Hsp90 with early unfolding intermediates of citrate synthase. *J Biol Chem*. 270: 7288–7294.
Lee GJ, Vierling E. (2000) A small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat-denatured protein. *Plant Physiol*. 122: 189-197.
de Leon, JH, Jones WA, Morgan DJW. (2004) Population genetic structure of *Homalodisca coagulata (*Homoptera: Cicadellidae), the vector of the bacterium *Xylella fastidiosa* causing PD in grapevines. *Ann Entomol Soc Am.* 97: 809-818.
Lindquist S, Craig EA. (1988) The heat-shock proteins. *Annu. Rev. Genet*. 22: 631-677.
Mircetich SM, Lowe SK, Moller WJ, Nyland G. (1976) Etiology of almond leaf scorch disease and transmission of the causal agent. *Phytopath.* 66: 1-24.
Nadeau K, Das A, Walsh CT. (1993) Hsp90 chaperonins possess ATPase activity and bind heat shock transcription factors and peptidyl prolyl isomerases. *J Biol Chem*. 268: 1479-1487.
Notredame C, Higgins DG, Heringa J. (2000) T-Coffee: A novel method for multiple sequence alignments. *J Mol Bio*. 302: 205-217
Page RDM. (1996) TREEVIEW: An application to display phylogenetic trees on personal computers. *Comp Appl Biosciences.* 12: 357-358.
Pelham HR. (1986) Speculations on the functions of the major heat shock and glucose-regulated proteins. *Cell.* 46: 959-961
Pelham H. (1988) Heat-shock proteins. Coming in from the cold. *Nature.* 332: 776-777.
Phillippe H, and Adoutte A. (1998) The molecular phylogeny of Eukaryota: solid facts and uncertainties. Pp. 25–56 in G. Coombs, K. Vickerman, M. Sleigh, and A. Warren, eds. Evolutionary Relationships Among Protozoa. Chapman & Hall, London.
Plesofsky-Vig N, Vig J, Brambl R. (1992) Phylogeny of the alphacrystallin- related heat-shock proteins. *J Mol Evol*. 35: 537–545.
Pooler MR, Hartung JS. (1995) Specific PCR detection and identification of *Xylella fastidiosa* strains causing citrus variegated chlorosis. *Curr Microbiol.* 31: 377-381.
Purcell AH. (1997) *Xylella fastidiosa,* a regional problem or global threat? *J Plant Pathol.* 79: 99-105.
Purcell AH, Saunders S, Hendson M, Grebus M, Henry M. (1999) Causal role of *Xylella fastidiosa* in oleander leaf scorch disease. *Phytopathol.* 89: 53-58.
Snutch TP, Heschl MF, Baillie DL. (1988) The Caenorhabditis elegans hsp70 gene family: a molecular genetic characterization. *Gene.* 64: 241-55.
Sobott F, Benesch JLP, Vierling E, Robinson CV. (2002) Subunit exchange of multimeric protein complexes: real-time monitoring of subunit exchange between small heat shock proteins by using electrospray mass spectrometry. *J Biol Chem*. 277: 38921–38929.
Sorensen JG, Kristensen GTN, Loeschcke V. (2003) The evolutionary and ecological role of heat shock proteins. *Ecol Lett*. 6: 1025–1037.
Sreedhar AS, Kalmar E, Csermely P, Shen YF. (2004) Hsp90 isoforms: functions, expression and clinical importance. *FEBS Lett*. 562: 11-15.
Stechmann A, Cavalier-Smith T. (2003) Phylogenetic analysis of eukaryotes using heat-shock protein Hsp90. *J Mol Evol*. 57: 408–419.
Takiya DM, McKamey SH, Cavichioli RR. (2006) Validity of *Homalodisca* and of *GWSS* as the name for glassy-winged sharpshooter (Hemiptera: Cicadellidae: Cicadellinae). *Annals Entomol Soc Am.* 99: 648-655.
Thompson JD, Higgins DG, Gibson TJ. (1994) "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice." *Nucleic Acids Res*. 22: 4673-4680.
Turner W, Pollard H. (1959) Life histories and behavior of five insect vectors of phony peach disease. *Technical Bulletin of the United States Dept Ag.* 28: 1188.
Young, DA. (1958) A synopsis of the species of *Homalodisca* in the United States. *Bulletin Brooklyn Entomol Soc.* 53: 7–13.

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ARE GLASSY-WINGED SHARPSHOOTER (GWSS) POPULATIONS REGULATED IN CALIFORNIA? LONG-TERM PHENOLOGICAL STUDIES FOR GWSS IN AN ORGANIC LEMON ORCHARD

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ABSTRACT
Glassy-winged sharpshooter (GWSS) population densities have been steadily declining over a 7.5 year period in organic lemons grown in an experimental study plot at UC Riverside Agricultural Operations. Peak adult GWSS populations in September 2009 were just 16% of those observed around August 2002. It is uncertain if egg parasitism, which has consistently averaged ~25% per year of GWSS egg masses is responsible for the observed decline. Density-dependent analyses of time series data are planned once data sets are large enough to provide greater insight into factors (i.e., parasitism [density-dependent mortality] or weather [density-independent mortality]) affecting GWSS population dynamics.

LAYPERSON SUMMARY
Glassy-winged sharpshooter (GWSS) populations in an organic lemon orchard in Riverside, southern California have been declining steadily since 2002. In September 2009, GWSS densities at their peak were only 16% of those observed at a similar peak in August 2002. This downward trend seems to have occurred in most of southern California but there are occasionally flare ups of GWSS as populations undergo localized outbreaks. The exact reasons for the significant decline in GWSS population densities is unknown, but could be due to the activity of natural enemies like egg parasitoids, or the weather, especially winter conditions, could be responsible. Consequently, the goal of this study is to figure out why GWSS populations have largely collapsed in southern California.

INTRODUCTION
Data collected from bi-weekly monitoring over the last 7.5 years from organic commercially-managed lemons at Agricultural Operations (Ag. Ops.), UC Riverside indicates that glassy-winged sharpshooter (GWSS) populations are declining steadily each year (**Figure 1**). It is uncertain whether parasitism of GWSS eggs by mymarid parasitoids is responsible for this downward population trend (**Figure 2**). In California, there is a guild of natural enemies attacking GWSS. The dominant parasitoid attacking GWSS in California is *Gonatacerus ashmeadi* followed by *G. morrilli*. *G. triguttatus* from Texas and *G. fasciatus* from Louisiana have been released in California, but widespread establishment and proliferation has not occurred. Other minor parasitoid species include *G. novofasciatus*, *Ufens* sp., and *Zagella* sp. Together, this guild of parasitoids provides an average of ~25% parasitism of GWSS eggs over the entire 7.5 yrs that this study site has been monitored. There are at least four possible reasons for low seasonal parasitism levels in California: (1) Competitive exclusion amongst members of the GWSS parasitoid guild is reducing effective biological control. (2) An extremely aggressive and efficacious natural enemy that can outcompete *G. ashmeadi* and completely dominate the system year round to the almost total exclusion of all current parasitoids has not been established in California and is needed for successful biological control of GWSS (this would require exploitation of non-GWSS hosts during long periods of host egg unavailability over winter). (3) The absence of resource subsidies such as nectar provided by flowering plants in agroecosystems may limit parasitoid efficacy because longevity and fecundity is significantly reduced when parasitoids can not access carbohydrates. Understory management may be an important cultural strategy to benefit GWSS parasitoids if it can be demonstrated not to enhance GWSS and *Xylella* populations. (4) Climate, in particular, prolonged cool periods over winter when GWSS eggs are unavailable probably has a severe affect on parasitoid reproductive success and the ability of *G. ashmeadi* and populations of other parasitoids to propagate through the winter. Long-term phenology studies which generate data similar to the project reported on here, can be used to tease out density-dependent and density-independent factors affecting population dynamics to elucidate factors affecting GWSS population growth.

OBJECTIVE
This project has one objective:
1. Conduct bi-weekly surveys of GWSS eggs, nymphs, and adults, and associated rearing of parasitoids from harvested egg masses from organic lemons at Ag. Ops., UC Riverside. These data will be analyzed to determine if density-dependent (e.g., natural enemies) or density-independent (e.g., winter temperatures and rainfall) influence observed GWSS population trends at the study site at UC Riverside.

RESULTS
The population monitoring study and measures of percentage parasitism clearly indicate that GWSS densities have continued to decline steadily at the long-term monitoring plot (**Figure 1**) and percentage parasitism have remained relatively constant

over this time period (**Figure 2**). Detection of density-dependent mortality from sequential census data such as that presented here is notoriously difficult and the results of analytical models differ in outcomes depending on assumptions made even when dummy data sets have been constructed to show density-dependent mortality. One of the major problems with these types of analyses is serial correlation, where densities at N_t directly influence the population at N_{t+1}. Recent developments in analyses of time series data, such as those we are collecting for GWSS are now providing much more robust tests that overcome autocorrelation problems. The Partial Rate Correlation Function (PRCF) is a relatively new statistical procedure specifically designed for time series analysis of biological populations to detect density dependent feed back. Literature searches so far indicate that PRCF is the best of the extant techniques for analyzing long-term population counts. Consequently, census data collected from GWSS monitoring will be subjected to PRCF once we have data for a minimum of 10 consecutive years to determine if density-dependent or density-independent feedback is responsible for observed fluctuations from generation to generation. Detection of density-dependent mortality will indicate that populations are being regulated, and could suggest that natural enemy populations are responsible. Currently, our data set is too short to determine if parasitoid activity is providing density-dependent mortality and is subsequently responsible for decreasing GWSS densities at the study site.

CONCLUSIONS

GWSS populations appear to be showing a steady annual decrease in numbers in an organic lemon orchard at UC Riverside. Percentage parasitism of GWSS eggs by mymarid parasitoids, in particular, *G. ashmeadi*, has remained relatively constant from year to year at ~25%. It is unknown if this level of parasitism is sufficient to have caused the steady decline in GWSS numbers observed over the past 7.5 years or whether climatic variables such as wet winters (e.g., 2006), or very cold and dry winters (e.g., 2007) suppressed GWSS population growth while warmer than normal spring periods (e.g., 2008) accounts for observed rebounds in GWSS populations.

REFERENCES CITED

Berryman, A, Turchin, P. 2001. Identifying the density-dependent structure underlying ecological time series. Oikos 92: 265-270.

Turchin, P. 1990. Rarity of density dependence or population regulation with lags? Nature 344: 660-663.

Turchin, P. 1995. Population Regulation: Old Arguments and a New Synthesis. In: Population Dynamics: New Approaches and Syntheses (Eds: N. Cappuccino & P.W. Price), pp.19-40. Academic Press, San Diego.

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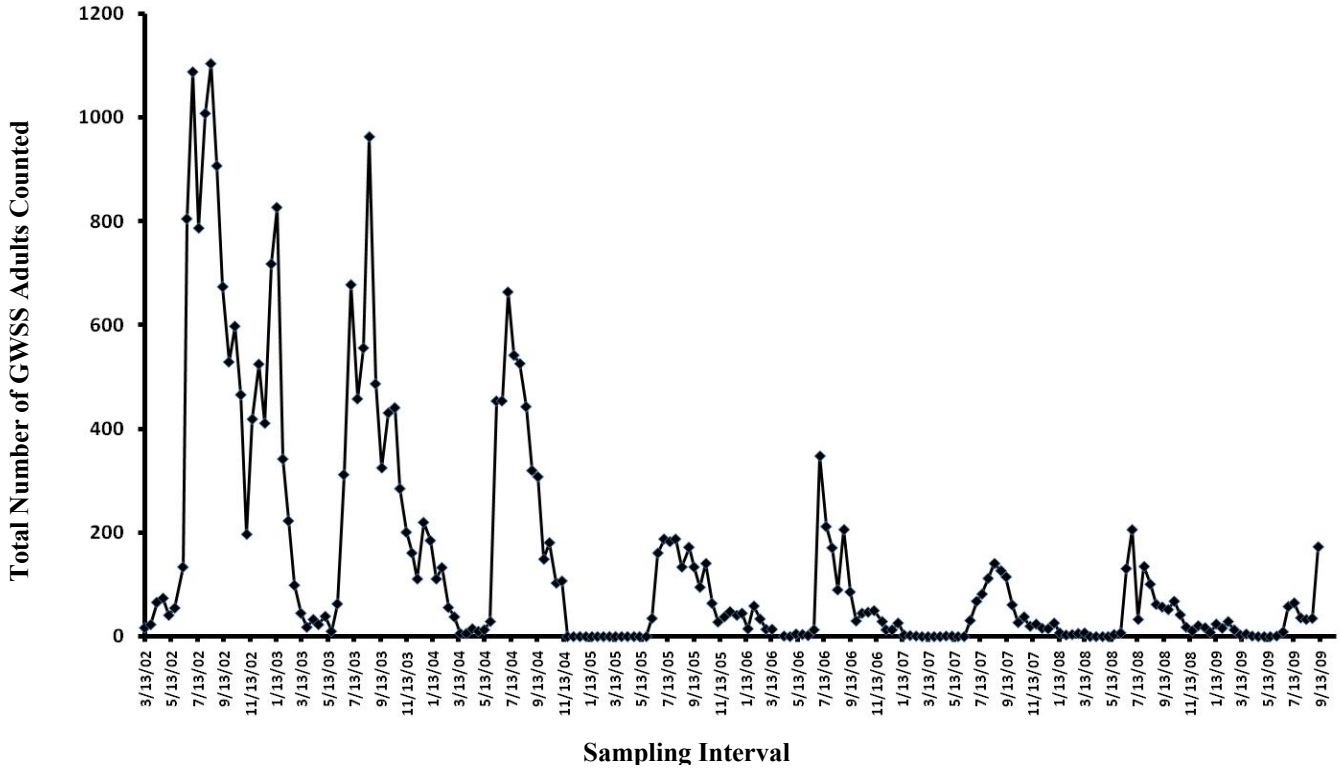

Figure 1. Phenology of adult GWSS in organic Eureka lemons. Data are total counts from timed five minute surveys made every two weeks of 10 mature lemon trees at UC Riverside Ag. Ops.

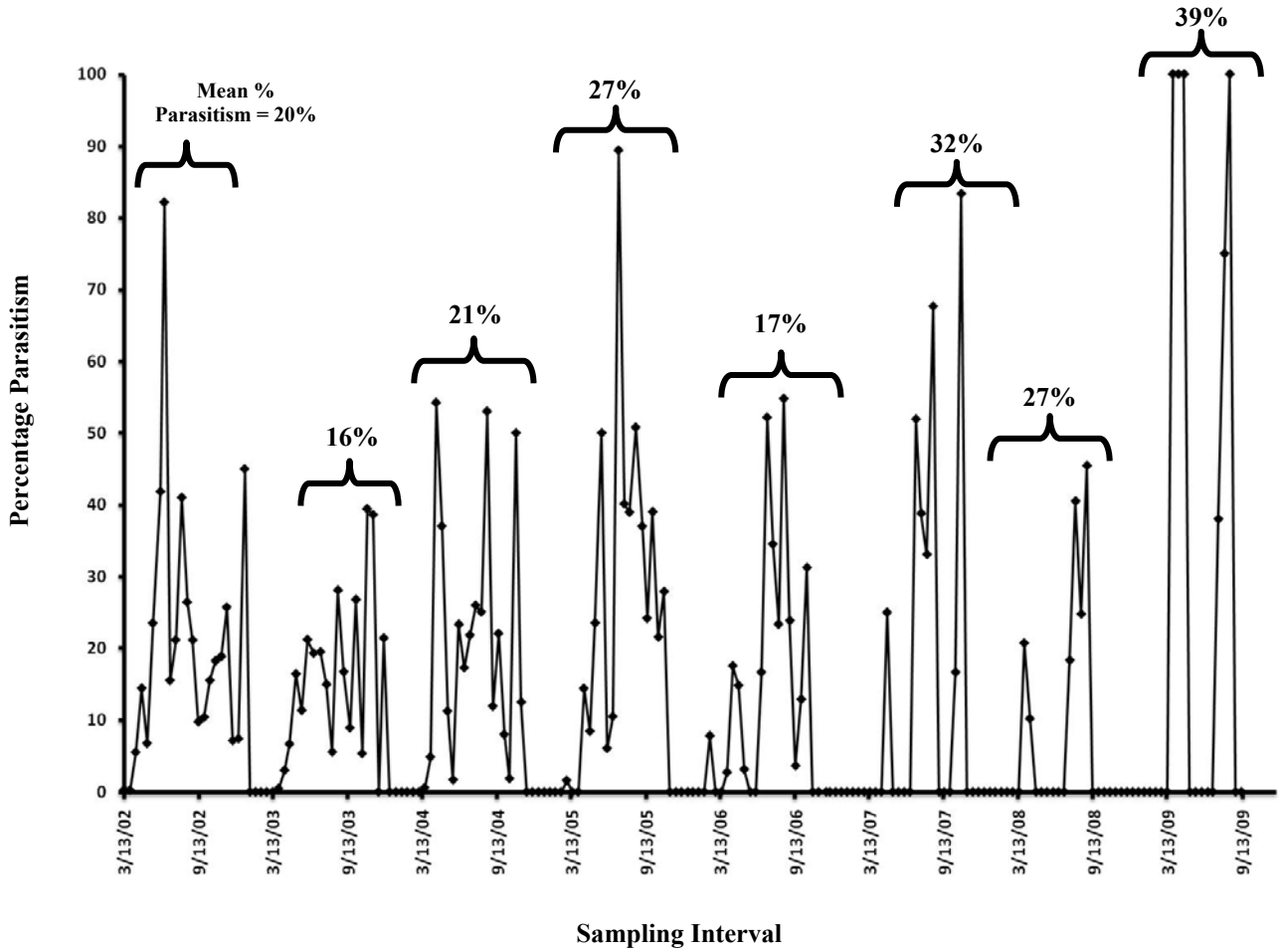

Figure 2. Percentage parasitism estimates of GWSS eggs in Eureka lemons. GWSS egg masses are collected from timed five minute surveys made every two weeks of 10 mature lemon trees at UC Riverside Ag. Ops. Harvested leaves are returned to the laboratory, the number of eggs per egg mass are counted and parasitoid emergence and species identity is determined. Percentage parasitism of GWSS eggs across all years has averaged ~ 25%.

EFFECT OF CONSTANT TEMPERATURE ON GLASSY-WINGED SHARPSHOOTER LIFE CYCLE

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ABSTRACT
The glassy-winged sharpshooter (GWSS) is a vector of *Xylella fastidiosa (Xf)*, the causal agent of Pierce's disease of grapevine. It is the most common leafhopper associated with vineyards in Texas, with the exception of the High Plains. In the Hill Country grape growing region of Central Texas, the insect overwinters in the adult stage. The earliest egg masses are laid in February and March when relatively cool temperatures prevail. Our interest was to determine the effect of ten constant temperatures on GWSS egg development and the effect of exposure periods of 6 to 120 hours in duration to subfreezing temperatures. The effect of temperature on nymphal growth and development was also measured. However, adverse artificial rearing conditions in the growth incubators negatively affected nymphal development which was twice as long as expected in the optimal temperature range under natural conditions. We were unable to draw conclusions without modifying the rearing conditions. The study on the effect of temperature on adult survival is underway and results are not available for this report.

LAYPERSON SUMMARY
The glassy-winged sharpshooter (GWSS) is an insect pest which can transmit the bacterium responsible for Pierce's disease of grapevine. The insect and the disease are commonly associated with vineyards in Texas and are the main limiting factor to grape production in the State. We studied the effect of ten temperatures on the life cycle of GWSS and the effect of short exposure periods to subzero temperatures on the eggs. Tests using the adults are underway. The data generated by these studies are critical to optimizing rearing techniques and for developing control strategies for grape growers.

INTRODUCTION
Grapevine hybrids of *Vitis vinifera* which are traditionally associated with the highest quality wines are varieties susceptible to various degrees of damage by Pierce's disease (PD). This disease is an incurable, debilitating and often fatal bacterial infection caused by *Xylella fastidiosa (Xf)* and disseminated by xylem fluid-feeding insects such as the glassy-winged sharpshooter (GWSS). In Texas we also recorded an additional 28 xylem fluid-feeding species associated with vineyards (Lauzière et al 2008). Many of these insect pests can vector the bacterium (Mitchell et al in press). PD is the most important limiting factor to grape production in Texas (Texas Pierce's Disease Task Force 2004). Funded by the U.S. Department of Agriculture, a statewide research program was initiated in 2002 to study vectors in their natural habitat, their interaction with cultivated vines and other vegetation, and investigate their biology in order to develop pest and disease management strategies.

GWSS is native to the Gulf Coast states of the USA (Young 1968). Indigenous populations of GWSS depend on the insects' ability to survive under the environmental conditions prevailing in the different grape growing regions of Texas. Among abiotic factors, temperature plays a major role influencing an insect's life cycle. Temperature interacts jointly with other factors such as humidity, food availability and light and since temperature is easily measured and controlled, it is common practice to examine its influence upon species of economic importance (Howe 1967). Thorough knowledge of the effects of temperature on development and survival, among other aspects of the biology and behavior of GWSS, is also critical for developing and optimizing rearing techniques under carefully regulated environmental conditions and for conducting field research aimed at developing control strategies.

OBJECTIVES
1. Determine the effect of constant temperatures on the life cycle of GWSS (embryo, nymph and adult)
2. Determine the effect of subzero temperatures on embryos and adults

RESULTS AND DISCUSSION
These studies were conducted at the Texas AgriLife Pierce Disease Research and Education Program facility in Fredericksburg, TX. Containerized *Euonymus japonica* were grown in a greenhouse setting and placed into cages with reproductively mature GWSS. The plants were monitored daily for leaves bearing egg masses. These were left *in situ* and enclosed in an organza pouch fastened with twist-ties. The egg masses were incubated in a growth chamber at the following temperatures: 12, 15, 18, 21, 24, 27, 30, 32.5, 35, and 37.5°C. Development of the eggs was monitored twice daily and emergence of nymphs was recorded. Undeveloped eggs were dissected under a stereomicroscope after 21 days and the total number of eggs was recorded. Because of the non linearity of development rates (the reciprocal of the developmental

period), only temperatures between 12 and 30˚C were used to compute the linear regression for embryonic development of GWSS. Nonlinearities of insect development at high temperatures justified the development of a nonlinear regression model. Therefore, embryonic development rate was fitted to the model of Logan et al. (1976). Embryonic survival was subjected to one-way analysis of variance (ANOVA) to test for temperature effect. When significant *F*-values were obtained, treatment means were discriminated using the Student Newman Keuls (SNK) test ($P < 0.01$).

We also studied the effect of freezing temperatures (-0.9± 0.21°C) on GWSS eggs exposed for 0, 6, 24, 48, 72 and 120 consecutive hours. After each specified time period, the plants bearing egg masses were transferred into a second growth chamber held at a constant 25°C and the eggs were monitored daily for nymphal emergence. Mortality was assessed as described above, 21 days after the mean emergence period when the eggs were assumed to be non-viable. We used a one-way ANOVA to estimate the effect of the exposure period on development time and survival. When significant *F*-values were obtained, treatment means were discriminated using the SNK test ($P < 0.01$).

Embryonic development of GWSS was successful to nymphal emergence between 15 and 35°C. Ultimate embryonic survival varied with temperature ($F = 3.09$; $df = 7, 156$; $P = 0.004$). The proportion of egg hatching was not significantly different for temperatures between 18 and 35°C and averaged 74.2% ± 2.9. At 15°C, percent survival was significantly lower with 43.8% ± 8.8 of the embryos developing into nymphs. Embryos continuously exposed to 12 or 37.5°C did not develop. The lower embryonic development threshold was calculated using a linear regression over the 15-30°C range and was estimated at 12.1°C. Using the Logan model and all temperatures tested, we determined that the optimal development temperature for GWSS eggs is 30.6°C. Embryonic development time decreased linearly between 15 and 30°C, ranging from 22.7 to 4.7 days.

Exposure to freezing temperatures delayed embryonic development for all exposure periods as compared to unexposed egg masses ($F = 201.17$; $df = 4, 384$; $P < 0.0001$). Eggs exposed to freezing temperatures for 6 and 24 hours required about nine days to complete their development, whereas embryogenesis of eggs treated for 48 hours and 72 hours took 11.8 and 13.2 days, respectively. Lethal effects occurred when eggs were kept below freezing for a consecutive 120 hours. This was also the only treatment which affected the plant. Percent survival was significantly different among the 24-120-hour exposure periods ($F = 16.99$; $df = 5, 113$; $P = 0.001$) with 26.6% survival measured after a 24-hour exposure down to 0% survival at 120 hours.

Nymphal development of GWSS fed black-eyed pea plants was studied by M. Sétamou at Weslaco in a similar fashion under controlled constant temperatures of 18, 21, 24, 27, 30, 34°C. In the optimal temperature range, development to adulthood required 45 days. In a previous study, nymphs reared by Lauzière and Sétamou (2009) at 25°C developed in 29.8 ± 0.7 days. We are critical of the data obtained from the temperature study and are concerned that food and light may have deeply affected the development of the nymphs reared under artificial light without sun light. Steps are being taken to correct our methodology so that we may draw conclusions that are more applicable to insect populations under natural conditions.

Temperature data summarized for the Hill Country of Central Texas indicated that the coldest months are usually December, January and February, with average temperatures of 8.8 to 15.1°C for the years 2003 to 2008. The warmest months are usually July and August which ranged 26.8 to 30.9°C in 2003-2008. During the past six years, the coldest year was 2004. Data from a previous study indicated that during the winter of 2004, temperatures in the vineyards remained below 0°C for a total of 100-140 hours (Lauzière et al. 2008). The winter of 2005 was not as cold as it was the previous winter, however, temperatures remained below zero for a total of 200-240 hours. On average, in this region, temperatures remain below zero for 8.6 ± 7.7 consecutive hours at a time and minimal temperatures of -2.8 ± 2.23°C (range -13.5-0.16°C) are recorded. It would be interesting to study this insect's development under cyclical temperature fluctuations in the low to freezing temperature range.

CONCLUSIONS

These studies showed that temperature had a strong influence on growth and development of GWSS. Embryonic development times decreased with increasing temperatures whereas mortality increased with increasing temperatures. For rearing purposes, temperatures of 28-30°C are optimum for the eggs and will yield the highest percentage of emerging nymphs.

Field data from 2005-2008 indicated that GWSS females actively produced eggs from February to September, with highest egg loads observed in March (13.8 ± 7.2 eggs/female; n = 155; (Lauzière 2008). During these months in Central Texas, relatively cool temperatures are usually observed which will affect glassy-winged sharpshooter development. However, the data suggest that egg development is possible and is correlated by field observations of the first generation of adults in early April.

Bioclimatic studies on insect hosts and their natural enemies can help explain their geographic distribution and also provide insight into the potential physiological limitations for their spread into other regions, either naturally or through unintentional translocations.

REFERENCES CITED

Howe, R. W. 1967**.** Temperature effects on embryonic development in insects. Annual review of Entomology 12: 15-42.

Lauzière, I. 2008**.** Reproductive behavior of a key vector of *Xylella fastidiosa* in Texas. In: Proceedings of the 2008 Pierce's disease reseach symposium, San Diego, CA. pp. 28-29.

Lauzière, I. and M. Sétamou. 2009**.** Suitability of different host plants for oviposition and development of *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae) and its implication on mass rearing. Annals of the Entomological Society of America. 120: 642-649.

Lauzière, I., S. Sheather and F. Mitchell. 2008**.** Seasonal abundance and spatio-temporal distribution of dominant xylem fluid-feeding Hemiptera in vineyards of Central Texas and surrounding habitats. Environmental Entomology 37: 925-937.

Logan, J. A., D. J. Wollkind, S. L. Hoyt and L. K. Tanigoshi. 1976. An analytical model for description of temperature dependent rate phenomena in arthropods. Environmental Entomology 5: 1130-1140.

Mitchell, F., J. Brady, B. Bextine and I. Lauzière**.** Seasonal increase of *Xylella fastidiosa* in Hemiptera collected from Central Texas vineyards. Journal of Economic Entomology (*in press*).

Texas Pierce's Disease Task Force. 2004**.** Does Texas hold the key to eradicating Pierce's disease? Wine Business Monthly 11: 34-38.

Young, D. A. 1968. Taxonomic study of the Cicadellidae (Homoptera: Cicadellidae). Part I Proconiini. U.S. National Museum Bulletin 261. Smithsonian Institution Press, Washington, D. C. 287 p.

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EXPANSION OF THE GLASSY-WINGED SHARPSHOOTER IN NORTH CAROLINA VINEYARDS AND ITS ASSOCIATION WITH THE MIMOSA TREE

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ABSTRACT
The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis)* was found in several North Carolina counties that were not previously reported between 2006 and 2009. Data from this four-year study showed that GWSS has been expanding to new sites; this movement appeared to start in the south coastal region and move to the northern and western parts of the state. Several GWSS specimens were found in Currituck Co. (in the most northeastern part of the state) in 2006 and none was found in Wake Co. (Piedmont) the same year in a vineyard that was heavily monitored since 2004, but GWSS appeared in this vineyard in 2007 and 2008. In addition, we found that GWSS preferred the mimosa tree, *Albizia julibrissin.* In areas close to creeks, large numbers of adults and nymphs were recorded in these plants. In the laboratory, this insect laid eggs and completed its life cycle feeding only on this plant. Currently, GWSS appears to have established populations in most of the Coastal Plain and in several areas of the Piedmont. However, GWSS was not detected in the Yadkin Valley (major vinifera growing region of NC) in the northwestern Piedmont, or the Mountain region of NC in 2009.

LAYPERSON SUMMARY
Data collected in this study showed that the glassy-winged sharpshooter (GWSS) has expanded its range to new sites in North Carolina. This movement appeared to start in the southern coastal region and move to the northern and western parts of the state. The GWSS was found in Currituck Co. (northeastern part of the state) but none was found in Wake Co. (Piedmont) in 2006 in a vineyard that was heavily monitored since 2004, but GWSS appeared in this vineyard in 2007 and 2008. In addition, we found that GWSS preferred the mimosa tree, as large numbers of adults and nymphs were recorded in these plants close to creeks. In the laboratory, this insect laid eggs and completed its life cycle feeding only in this plant. Currently, GWSS appears to be established in most of the Coastal Plain, and several areas of the Piedmont. However, GWSS was not been detected in the Yadkin Valley in the northwestern Piedmont (major vinifera growing region of NC) or the Mountain region of NC in 2009.

INTRODUCTION
Leafhoppers are vectors of *Xylella fastidiosa* (*Xf*), the causal agent of Pierce's disease (PD) in European grapes (*Vitis vinifera*). The glassy-winged sharpshooter (GWSS) has become a well known subject of study since its introduction to California. There, the grape growers faced a dire situation after the arrival of GWSS - vines infected with *Xf* increased and the disease became widespread. Reports comparing the transmission of *Xf* by GWSS with native Californian species such as *Graphocephala atropunctata* showed a lower transmission capacity of the former (Hill and Purcell 1995); however, its dispersion capacity (ability to fly long distances) facilitated the expansion of the disease.

In *vinifera* growing areas of NC, four species of sharpshooters were reported prior to the beginning of this study (Villanueva et al. 2007). The presence of the GWSS may increase the incidence of PD, and further limit development of the vinifera industry in North Carolina. In a preliminary study, we observed that the mimosa tree (*Albizia julibrissin*) was apparently a good host of GWSS and leafhoppers were monitored with yellow sticky traps placed in several vineyards of NC and in areas where mimosa trees grows.

OBJECTIVES
1. To evaluate the distribution of GWSS in North Carolina.
2. To study the importance of *Albizia julibrissin* as preferred host of GWSS.

RESULTS AND DISCUSSION

GWSS was first reported in NC in Pender Co. in 2002 (David Stephan, personal communication) prior to this study (**Figure 1**). Pender Co. is located in the southeastern Coastal Plain. In 2006, GWSS was found on yellow sticky traps collected from Currituck Co. (the most northeastern county of NC), which indicates that GWSS has moved to the north. Myers et al (2007) using yellow sticky traps, did not collect any GWSS in two Piedmont vineyards, one located in Wake Co. and the second in Alamance Co. in 2004 and 2005 (**Figure 1**). However, in this study we sampled intensively (>12 traps/vineyard) the same vineyards from 2006 to 2009 and GWSS was found in large numbers in 2007 and 2008 in the Wake Co. vineyard and in addition, live specimens were collected in the NC State University campus in 2009 (in the same county). Also, one GWSS was found in Alamance Co. in August 2009 (**Figure 1**). These results indicate that GWSS has moved from the eastern NC to the west over the past three years. GWSS could have been present in the Coastal Plain counties before this study started in 2006. This area is where muscadine grapes are grown and large numbers of GWSS were detected in traps near muscadine vineyards from mid-June to October in 2007 and 2008. Additionally, they were more abundant in muscadine vineyards than *Oncometopia orbona* the most well distributed sharpshooter in NC. The cause of this migration may be the warmer temperatures which have been observed in recent years (Anas et al. 2008).

We also sampled areas where *Albizia julibrissin* grows; these sites were near vineyards or beside roads and creeks. In most cases we captured GWSS, especially in areas close to creeks. More GWSS were captured in traps hung close to young *A. julibrissin* than plants of *Lonicera albiflora* (honeysuckle), a *Rhus* sp. (sumac) and *Rubus* sp. (blackberry) growing nearby, cherry (*Prunus* sp.), wild grape (*Vitis* sp.) or an old *A. julibrissin* tree (20 ft tall) (**Figure 2a**). The young *A. julibrissin* plants were cut every year from the base and trunks by highway maintenance crews and can grow 2.5 to 3.5 m from April to September. Also, by mid-June large numbers of *O. orbona* were caught in traps in old *A. julibrissin* (~20 ft height) plants. The reason for this is unknown but this plant may be important not only as preferred host of GWSS but it may be a temporary host of *O. orbona* during this time of the year. However, in live counts, GWSS was generally found in greater numbers compared with other species in *A. julibrissin* plants (**Figure 3**). In addition, GWSS females -placed in cages containing six-eight month old *A. julibrissin* plants- were able to laid eggs, and complete their development on these plants alone.

CONCLUSION

In this study we found that GWSS has expanded to new areas of North Carolina. This insect might have been undetected along the coast and southern part of the state for many years. However, in spite of intensive monitoring, it was not detected in the central part of the state until 2007 when we captured it in a vineyard in Wake Co. and 2009 in Alamance Co. Many species of leafhoppers that are vectors of *Xf* are endemic to NC, but GWSS may cause *Xf* to spread more rapidly. Additionally, we found that the mimosa tree is a preferred host of GWSS. GWSS was able to lay eggs, and completed its life cycle on this plant. Also, direct visual counts and trap catches showed a preference for it compared to the surrounding vegetation. Additionally, large numbers of *O. orbona* were found in old *A. julibrissin* trees.

REFERENCES CITED

Anas, O., U. J. Harrison, P. M. Brannen, and T. B. Sutton. 2008. The effect of warming winter temperatures on the severity of Pierce's disease in the Appalachian Mountains and Piedmont of the southeastern United States. Plant Management Report Online, *On the web*: http://www.plantmanagementnetwork.org/sub/php/research/2008/pierces/.

Hill, B. L. and A. H. Purcell. 1995. Acquisition and retention of *Xylella fastidiosa* by an efficient vector, *Graphocephala atropunctata*. Phytopathology. 85:209-212.

Myers, A.L., T. B. Sutton, J.A. Abad, and G.G. Kennedy. 2007. Pierce's disease of grapevines: identification of the primary vectors in North Carolina. Phytopathology. 97:1440-1450.

Villanueva, R. T., G. G. Kennedy, and T. B. Sutton. 2007. Survey of leafhoppers on grapes in the Piedmont and Mountain regions of North Carolina, pages 62-64. In: T. Esser. Ed. *Pierce's disease control program: Symposium Proceedings*, 293 pp.

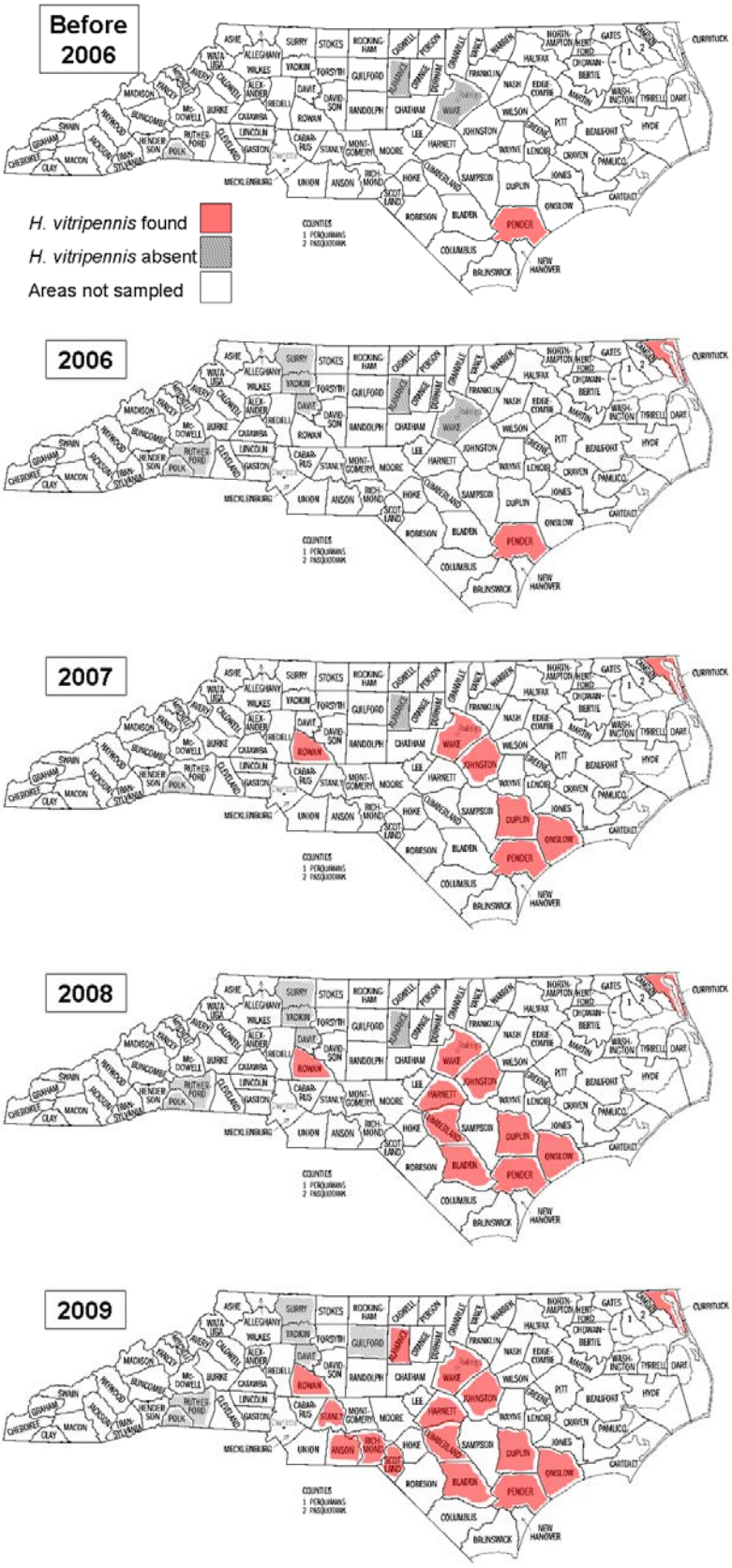

Figure 1. North Carolina maps showing counties where GWSS were present, absent, and not sampled.

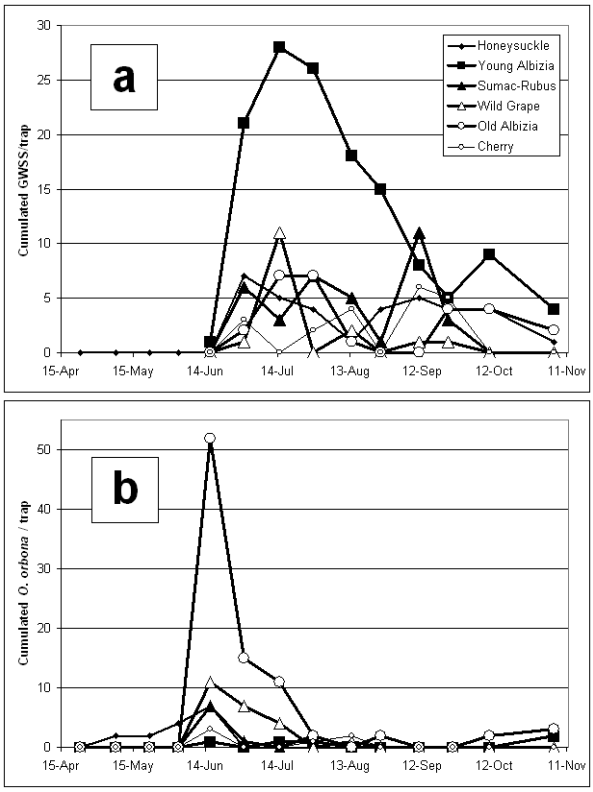

Figure 2. Cumulated numbers of (a) *GWSS* (GWSS) and (b) *Oncometopia orbona* caught in yellow sticky traps placed in honeysuckle, sumac- Rubus, wild grape, cherry, and old and young *A. julibrissin* trees in 2008. Traps were replaced every 2 weeks.

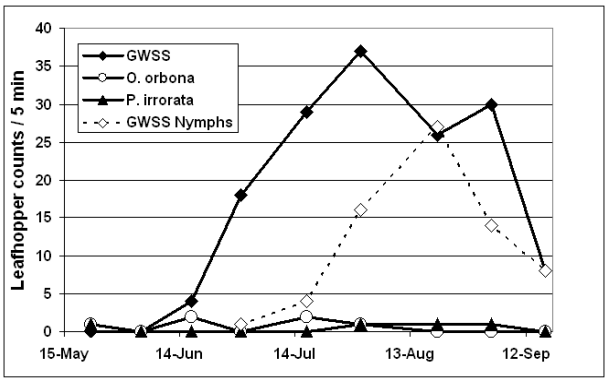

Figure 3. Numbers of GWSS (GWSS adult or nymphs), *Oncometopia orbona*, and *Paraulacizes irrorata* found during 5-min interval counts in *Albizia julibrissin* plants in 2007

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